SHOUT
SHARE
IT

MARINE BIOLOGICAL LABORATORY
WOODS HOLE
LIBRARY
MASS

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

HAROLD C. BOLD, University of Texas
FRANK A. BROWN, JR., Northwestern University
JOHN B. BUCK, National Institutes of Health
T. H. BULLOCK, University of California, Los Angeles
E. G. BUTLER, Princeton University
J. H. LOCHHEAD, University of Vermont
ARTHUR W. POLLISTER, Columbia University
C. L. PROSSER, University of Illinois
MARY E. RAWLES, Carnegie Institution of Washington
WM. RANDOLPH TAYLOR, University of Michigan
A. R. WHITING, University of Pennsylvania
CARROLL M. WILLIAMS, Harvard University

DONALD P. COSTELLO, University of North Carolina
Managing Editor

VOLUME 114
FEBRUARY TO JUNE, 1958

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 1 and September 1, and to Dr. Donald P. Costello, P.O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa., under the Act of August 24, 1912.

LANCASTER PRESS, INC., LANCASTER, PA.

CONTENTS

No. 1. February, 1958 PAGE

GRANT, WILLIAM C., JR., AND JOAN A. GRANT
Water drive studies on hypophysectomized efts of Diemyctylus viridescens 1
GRINNELL, ALAN D., AND DONALD R. GRIFFIN
The sensitivity of echolocation in bats 10
HARVEY, WILLIAM R., AND CARROLL M. WILLIAMS
Physiology of insect diapause. XI. Cyanide-sensitivity of the heartbeat of the Cecropia silkworm, with special reference to the anaerobic capacity of the heart 23
HARVEY, WILLIAM R., AND CARROLL M. WILLIAMS
Physiology of insect diapause. XII. The mechanism of carbon monoxide-sensitivity and -insensitivity during the pupal diapause of the Cecropia silkworm 36
HYMAN, LIBBIE H.
Notes on the biology of the five-lunuled sand dollar 54
LOOSANOFF, V. L.
Some aspects of behavior of oysters at different temperatures 57
MCDONALD, BARBARA BROWN
Quantitative aspects of deoxyribose nucleic acid (DNA) metabolism in an amicronucleate strain of Tetrahymena 71
MARUYAMA, K.
Contractile protein from crayfish tail muscle 95
HYMAN, LIBBIE H.
The occurrence of chitin in the lophophorate phyla 106

No. 2. April, 1958

ALLEN, ROBERT D., AND EDWARD C. ROWE
The dependence of pigment granule migration on the cortical reaction in the eggs of Arbacia punctulata 113
BUCK, JOHN
Cyclic CO_2 release in insects. IV. A theory of mechanism 118
CHACE, FENNER A., JR.
A new stomatopod crustacean of the genus Lysiosquilla from Cape Cod, Massachusetts 141
CHRISTENSEN, AAGE MØLLER, AND JOHN J. MCDERMOTT
Life-history and biology of the oyster crab, Pinnotheres ostreum Say . . 146
CLARK, A. M., AND M. J. PAPA
Some effects of oxygen upon the white pupae of Habrobracon 180

OEC 12

GANAROS, ANTHONY E.
On development of early stages of Urosalpinx cinerea (Say) at constant temperatures and their tolerance to low temperatures 188
HSIAO, SIDNEY C., AND HOWARD BOROUGHS
The uptake of radioactive calcium by sea urchin eggs. I. Entrance of Ca^{45} into unfertilized egg cytoplasm 196
JOHNSON, T. W., JR.
A fungus parasite in ova of the barnacle Chthamalus fragilis denticulata . . 205
LYNCH, WILLIAM F.
The effect of x-rays, irradiated sea water, and oxidizing agents on the rate of attachment of Bugula larvae 215
MALAMED, SASHA
Gastrular blockage in frogs' eggs produced by oxygen poisoning 226
MAZIA, DANIEL
The production of twin embryos in Dendraster by means of mercaptoethanol (monothioethylene glycol) 247
TWEEDELL, KENYON S.
Inhibitors of regeneration in Tubularia 255

NO. 3. JUNE, 1958

BRYAN, JOHN H. D., AND JOHN W. GOWEN
The effects of 2560 r of x-rays on spermatogenesis in the mouse 271
COSTLOW, JOHN D., JR., AND C. G. BOOKHOUT
Larval development of Balanus amphitrite var. denticulata Broch reared in the laboratory 284
DAVIS, H. C.
Survival and growth of clam and oyster larvae at different salinities . . . 296
EKBERG, DONALD R.
Respiration in tissues of goldfish adapted to high and low temperatures 308
FINGERMAN, MILTON, AND MILDRED E. LOWE
Stability of the chromatophorotropins of the dwarf crayfish, Cambarellus shufeldti, and their effects on another crayfish 317
GROSS, WARREN J.
Potassium and sodium regulation in an intertidal crab 334
MCFARLAND, WILLIAM N., AND FREDERICK W. MUNZ
A re-examination of the osmotic properties of the Pacific hagfish, Polistotrema stouti 348
MOULTON, JAMES M.
The acoustical behavior of some fishes in the Bimini area 357
PROVASOLI, L.
Effect of plant hormones on Ulva 375
RUGH, ROBERTS
The so-called "recovery" phenomenon and "protection" against x-irradiation at the cellular level 385
YOUNG, RICHARD S.
Development of pigment in the larva of the sea urchin, Lytechinus variegatus 394

Vol. 114, No. 1 February, 1958

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

MARINE BIOLOGICAL LABORATORY
LIBRARY
WOODS HOLE, MASS.

WATER DRIVE STUDIES ON HYPOPHYSECTOMIZED EFTS OF DIEMYCTYLUS VIRIDESCENS. PART I. THE ROLE OF THE LACTOGENIC HORMONE

WILLIAM C. GRANT, JR. AND JOAN A. GRANT

Department of Biology, Williams College, Williamstown, Massachusetts

It is well known that following metamorphosis the eastern, spotted newt *Diemyctylus viridescens* passes into a terrestrial or red eft stage which lasts from three to four years before the animals migrate to water where they become sexually mature. In certain parts of Long Island and in the Woods Hole area, however, Noble (1926, 1929) found that the eft stage failed to develop. There has been considerable interest in the role played by the endocrine glands in the events associated with the migration of efts from land to water. Adams (1932) was able to induce adult skin texture and pigmentation in efts injected with an anterior lobe preparation (phyone), while Dawson (1936) showed that pituitary preparations administered to efts brought about the maturation of the lateral line system. The studies of Reinke and Chadwick (1939) demonstrated that efts receiving implants of whole adult pituitaries or anterior lobes voluntarily migrated to water from 2 to 6 days following treatment. The test animals acquired a smooth, moist skin and showed a tendency toward the olive pigmentation of the adult. In certain cases keeling of the tail was evident after an extended period. The thyroids showed some stimulation as the result of the implants but the gonads remained unaffected. Molting usually occurred on the second to fourth day following implantation. Gonadectomized efts molted and entered water from 4 to 8 days after implantation of adult pituitaries according to Reinke and Chadwick (1940). Thyroidectomized individuals and animals which had been both thyroidectomized and gonadectomized were forced to water following similar treatment, although in these cases molting was abnormal with pieces of cornified epithelium sloughing off in patches after the animals had assumed the aquatic habitat.

The failure of thyroidectomized efts to undergo a normal molt is understandable in the light of investigation by Adams and her co-workers. Adams *et al.* (1932) and Adams and Grierson (1932) have shown that a pituitary-thyroid relationship is necessary for proper molting. Changes in cutaneous circulation, rather than the stimulation of secretion of the cutaneous glands, may be of major importance to the molting process. According to Chadwick (1948), however, the thyroid exerts a direct effect on molting by the stimulation of the inter-papillary skin glands. An increased incidence in molting, noted by Chadwick and Jackson (1948) following

Copyright © 1958, by the Marine Biological Laboratory

the injection of efts with prolactin, may have been due to the stimulation of cell division in the epidermis.

Chadwick (1940a) induced water drive in efts ranging from 60 to 95 mm. in length with injections of Antuitrin G (Parke Davis). This preparation was less effective than implants of adult newt pituitaries, as it failed to induce water drive in smaller animals or those which had been thyroidectomized. Prolactin was identified with the active water drive principle of the anterior lobe by Chadwick (1940b). Injections of 14 to 20 mg. of prolactin (Eli Lilly unidentified lot) caused water drive in all normal, thyroidectomized and gonadectomized efts within a period of 10 days. Chadwick (1940c, 1941) obtained the migration of efts to water following intramuscular and intraperitoneal implants of hypophyses of a variety of vertebrates such as the water snake (Natrix), the domestic fowl and several genera of urodele and anuran amphibians. That the water drive reaction may be more complex is indicated by Dr. Richard W. Payne (unpublished data) who obtained positive results after the injection of a wide number of pituitary preparations, several of which showed negative prolactin activity by the pigeon crop assay. Tuchmann-Duplessis (1948, 1949) has shown that the administration of 60–120 Riddle-Bates units of prolactin to the land stage of *Triturus cristatus* and *T. alpestris* resulted in the migration of the animals to water and the assumption of pigmentation and morphological characters associated with the aquatic, reproductive phase. The cloaca became enlarged while the gonads and prostate became active. Prolactin administered to castrated males produced only water drive and color changes.

The results reported above indicate that prolactin is probably the active principle concerned with the initiation of water drive and that the thyroid, while not necessary to this reaction, facilitates the process by conditioning normal molting. Nevertheless, the situation remains confused, considering that a number of hormone preparations other than prolactin have produced water drive activity, and it is not clear which of the various phenomena accompanying migration (pigment changes, etc.) are stimulated directly by the water drive factor and which result from endogenous release of other endocrine substances through stimulation of the pituitary. The present investigation is part of an extended study seeking to clarify the complex situation involved in the transformation of the terrestrial eft to the aquatic adult. The use of hypophysectomized test animals has been necessary in order to rule out the endogenous release of prolactin itself by a specific testing agent or nonspecific "shock" effect and to eliminate synergic reaction within the pituitary. Grant and Grant (1956) have previously indicated that prolactin causes water migration and skin changes in hypophysectomized efts but none of the other changes toward the adult condition.

"Water drive" is used below to indicate the actual migration of efts to water and "water drive syndrome" to designate migration plus associated morphological changes, etc. The term "drive" is used loosely and is not necessarily meant to imply the operation of precise directive factors.

The authors wish to express their indebtedness to Dr. Grace E. Pickford, Bingham Oceanographic Laboratory, Yale University, for suggesting the project and for furnishing many of the preparations used. We are further indebted to

Dr. C. H. Li of the Hormone Research Laboratory, University of California, for the donation of a quantity of his highly purified prolactin used in the tests. This investigation was sponsored, in part, by a grant from the Mearl Corporation of New York City, through the kindness of Mr. Harry E. Mattin and Dr. Leon M. Greenstein, while a generous Grant-in-Aid from the Signa Xi-RESA Research Fund helped finance investigations through the past year. Our thanks are extended to Williams College and to Dr. S. A. Matthews, Chairman of the Department of Biology, for laboratory space and numerous facilities used during this investigation.

Materials and Methods

Efts were collected near Lyme Center, New Hampshire; Quechee, New York; Honesdale, Pennsylvania, and Williamstown, Massachusetts. Animals from different localities were segregated and kept in plastic boxes, 15–20 animals per box, on a thick bed of moss (usually Polytrichum). Room temperature ranged from 21°–24° C. Illumination was that of the room except for a few hours each day when the containers were subjected to direct illumination from an overhead lamp. This procedure aided the growth of the moss, the presence of which seems to be extremely helpful in keeping animals healthy. Attempts made to keep efts on a floor of wet, cellulose sponge proved quite unsuccessful. Animals were fed Enchytraeus worms and blowfly larvae. Some test animals were kept without feeding for extended periods in a refrigerator at approximately 5° C. Hypophysectomized efts are particularly susceptible to infection, but 1% solutions of potassium bichromate or malachite green diluted 1:15,000 have proved efficient prophylactic agents.

The total length of experimental animals ranged from 40 to 74 mm. with weight varying from 0.5 to 1.4 gm. The probable age of such animals was from 1 to 2.5 years and all were well removed from the naturally occurring aquatic phase of their cycle. Snout-vent measurements, which have proved an accurate standard in herpetological work, were also recorded.

Animals were anesthetized in a 0.5% solution of chloretone and hypophysectomized with the aid of a suction pipette attached to an aspiration unit. At the termination of the experiments hypophysectomy was verified histologically on most specimens. Two weeks after hypophysectomy subcutaneous injections were made with a 27-gauge, Huber point needle. In general, injections were made every other day at various concentrations of solution, but a constant volume of 0.1 cc. at each injection was maintained. Water drive responses were studied in containers possessing equal areas of land and water. The time taken for the assumption of the aquatic habitat, the number of molts and the duration of the aquatic phase of life were recorded for all individuals as far as possible.

The various preparations used in the injections were made up in a standard amphibian Ringer's, with controls receiving the same volume of saline as experimental animals. The following pituitary preparations were tested: FSH (swine, Armour Lot No. K45208R), LH (sheep, Armour Lot 227–80), TSH (Armour Lot 317–51), Antuitrin S (Parke Davis Lot P459D), ACTH (hog, Armour Lot K 52204), posterior pituitary preparation (hog, Lot 503), GH (beef growth hormone, Wilhelmi Lot B168), prolactin (Sheep, Schering Lot 4g Hyex 4, Armour sheep Lot 759–CCC and the highly purified sheep preparation of Li), MSH

TABLE 1

Results of water drive studies following the treatment of land phase Diemyctylus viridescens with various pituitary preparations

Treatment	Hypophysectomy	Wt., gm.	Length, mm. Total/Standard	Total dose, mg.	Results	Molting
*FSH Armour**	–	0.55	55/26	8	9 days to water	+
	–	0.80	55/29	8	10 days to water	+
	+	1.20	67/36	8	partial response	abnormal
	+	1.40	69/36	8	8 days to water	abnormal
	+	0.85	67/34	0.8	negative response	–
	+	0.72	63/32	0.8	negative response	–
	+	0.47	56/29	0.8	negative response	–
LH Armour	–	0.81	56/33	8	negative response	–
	–	0.47	48/23	8	negative response	–
	+	1.10	73/36	8	negative response	–
	+	1.12	68/37	8	negative response	–
GH Wilhelmi	+	1.10	73/38	0.8	negative response	+
	+	0.53	60/29	0.8	negative response	–
	+	0.53	59/32	0.8	negative response	–
	+	0.72	63/33	0.8	negative response	–
	+	0.74	63/32	0.8	negative response	–
	+	0.53	51/31	0.8	negative response	–
ACTH Armour	+	0.62	57/26	0.8	negative response	–
	+	0.71	64/32	0.8	negative response	+
	+	0.65	59/32	0.8	negative response	–
	+	0.67	62/34	0.8	negative response	–
	+	0.68	64/33	0.8	negative response	–
A corticotropin (Li)	+	0.84	65/35	0.8	negative response	–
Posterior pituitary Armour	+	1.22	72/37	0.8	negative response	–
	+	0.68	58/32	0.8	negative response	–
	+	0.50	52/31	0.8	negative response	–
	+	0.62	54/31	0.8	negative response	–
TSH Armour	+	0.61	58/32	0.8	negative response	–
	+	0.74	61/34	0.8	negative response	+
	+	0.84	65/35	0.8	negative response	+
	+	0.57	54/33	0.8	negative response	+
	+	0.62	60/33	0.8	negative response	+
Antuitrin S Parke & Davis	–	0.65	52/30	6	dead 7th day	–
	–	0.72	53/35	4	dead 5th day	–
MSH Armour	+	0.88	66/35	2	negative response	–
	+	0.47	51/28	2	negative response	–
	+	0.55	55/30	2	negative response	–
Intermedin (Li)	+	0.60	58/39	0.8	negative response	–

* All animals receiving 8 mg. of FSH showed a tendency toward the olive pigmentation of the adult.

(melanophore stimulating hormone, Armour Lot R 527225). According to Steelman *et al.* (1953), the assay for the FSH preparation shows it to be contaminated with 0.5 I.U. of prolactin per mg., a fact which is of importance in interpreting the results given below.

RESULTS

(*a*) *Various mammalian pituitary preparations*

The detailed results of this series of injections are given in Table I. LH, TSH, ACTH, MSH, posterior pituitary, GH and Antuitrin S failed to induce water drive in any of the animals tested. One animal given 0.8 mg. of ACTH-free Intermedin (Li) also gave a negative response. Most efts treated with TSH underwent a normal molt following injections, while one eft of the GH series and one of the ACTH series showed this reaction. All other preparations failed to produce normal molts in hypophysectomized individuals with the result that the

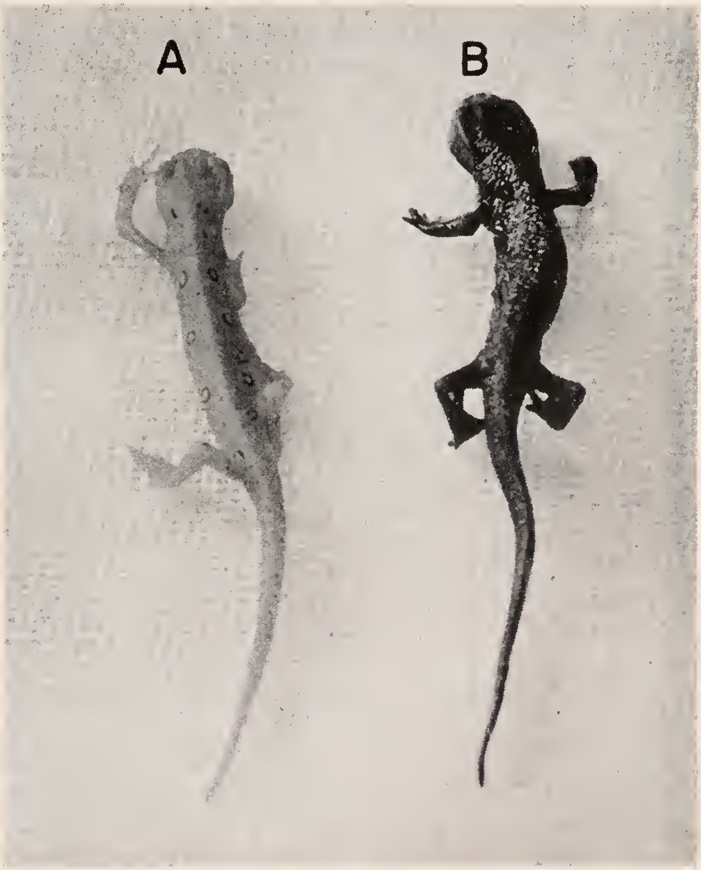


FIGURE 1. A normal eft (A) is shown beside a hypophysectomized animal (B), which having failed to molt is covered with a thick layer of cornified epithelium.

TABLE II

Results of water drive studies following the treatment of land phase Diemyctylus viridescens with prolactin

Treatment	Wt., gm.	Length, mm. Total/Standard	Total dose, mg.	Effective dose I.U.*	Results	Molting
Non-hypophysectomized *Schering prolactin* 30 I.U./mg.	0.54	50/28	8	240	7 days to water	+
	0.64	51/29	8	240	7 days to water keeling of tail and olive pigmentation	+
Hypophysectomized *Armour prolactin* 25–30 I.U./mg.	1.12	74/39	8	216	8 days to water	abnormal
	0.41	56/29	8	162	6 days to water	–
	0.61	63/33	0.8	23	10 days to water	abnormal
	0.43	55/28	0.8	23	8 days to water	–
	0.76	57/31	0.8	23	7 days to water	abnormal
	0.80	60/29	0.08	—	partial response	abnormal
Hypophysectomized *Prolactin (C.H.Li)* 35 I.U./mg.	0.66	57/32	0.4	14	10 days to water	–
	1.14	69/36	0.4	10.5	5 days to water	–
	0.63	59/32	0.4	10.5	5 days to water	abnormal
	0.75	65/34	0.4	7	4 days to water	abnormal
	0.59	58/32	0.4	10.5	5 days to water	–
	0.48	55/29	0.4	—	Dead on 3rd day	
	0.73	60/32	0.4	—	Dead on 4th day	
	0.80	62/34	0.4	14	7 days to water	–
	0.71	57/31	0.4	14	8 days to water	abnormal
	0.35	40/26	0.4	14	7 days to water	abnormal
	0.60	57/30	0.4	14	7 days to water	–
	0.61	54/29	0.4	14	8 days to water	abnormal
	0.97	69/36	0.4	10.5	6 days to water	abnormal
Hypophysectomized *Prolactin (C.H.Li)* 35 I.U./mg.	0.87	58/34	0.04	1.4	8 days to water	–
	0.92	64/35	0.04	1.4	10 days to water	–
	0.63	57/29	0.04	1.4	8 days to water	abnormal
	0.82	65/34	0.04	1.4	8 days to water	–
	0.44	50/27	0.04	1.4	8 days to water	abnormal
	0.78	70/35	0.04	1.05	6 days to water	–

* Effective dose is estimated as the amount of prolactin efts had received at the time of their assumption of an aquatic habitat.

efts rapidly became covered with a thick, black layer of cornified epithelium until even the eyes were obscured (Fig. 1).

Both normal and hypophysectomized efts receiving a total of 8 mg. of FSH showed water drive activity from 8 to 10 days following the initial injections. One animal failed to give a complete reaction and was in and out of water for several weeks before returning permanently to land. In these animals molting was abnormal with the skin sloughing off in irregular patches after the efts had entered water. There was a trend in the pigmentation of all individuals toward the adult olive, though this was more marked in the non-operated efts. It is interesting that tests for water drive in animals receiving 0.8 mg. of FSH were completely negative, and that molting failed to occur.

(*b*) *Tests with prolactin*

Two unhypophysectomized animals receiving 8 mg. (240 I.U.) of Schering prolactin migrated to water on the seventh day following the initial injections and within a few weeks had acquired many features associated with the water drive syndrome (*i.e.,* smooth, moist skin, olive pigmentation and keeling of the tail). Other prolactin preparations were injected into hypophysectomized efts in doses varying from 8.0 to 0.04 mg. as shown in Table II. In all cases where death did not occur before the injections were completed, the tests were positive. The animals assumed the aquatic habitat from 4 to 10 days following the first injection. It should be noted that several animals migrated before all injections had been completed and it is therefore desirable to give results in terms of the effective dose (*i.e.,* the dose animals had received at the time of the water-drive response) rather than total dose. The range in effective dose was from 216 to 1.05 I.U.

The water drive reaction is very positive as animals giving the reaction remain completely submerged, take food under water and will immediately return to water if placed on land. One eft receiving 0.08 mg. (2.3 I.U.) failed to give the complete reaction but migrated alternately between land and water over the time observed. Records on the duration of water drive are far from complete as most test animals died before leaving water. However, in a number of cases animals actually did return to land after periods varying from two to five weeks.

It is of particular significance to note that whereas all hypophysectomized animals showed positive water drive in response to treatment with prolactin, they failed to assume the olive pigmentation and tail keel associated with the water drive syndrome. When molting occurred it was abnormal, but beneath the patches of thickened corneum the smooth, moist skin retained the orange pigmentation of the eft and showed no tendency toward the adult olive. No keeling of the tail was apparent in any individual even after extended periods in water.

Conclusions

The primary concern of the present investigation was to determine as precisely as possible the endocrine factor responsible for water drive in the red eft. From the results reported above we are in agreement with Chadwick (1940c) that prolactin is the active principle. All animals treated with this substance migrated to water and assumed a smooth skin texture similar to that of the adult. As tests were conducted on hypophysectomized efts the possibility of hypophyseal synergy or endogenous release must be ruled out. All other pituitary preparations administered gave a negative reaction with the exception of the 8-mg. dose of FSH. This is understandable, as the assay for the gonadotropin shows it to be contaminated with lactogenic hormone. Both the Armour prolactin and the homogeneous preparation of Li produced positive results in animals receiving as little as 2.3 to 1.4 I.U. per effective dose. The 4 I.U. of prolactin contained in the FSH were therefore quite sufficient to initiate water drive. No minimum dosage level for the water drive reaction has yet been established but the failure of 0.8 mg. FSH to elicit positive results may indicate it to be about 0.4 I.U. Though there was some variability in the time animals responded to treatment with prolactin, there is at present no indication of a dose-response relationship and it is suggested that the reaction may follow the all-or-none principle.

Reinke and Chadwick (1940) have shown that the thyroid and gonads are not directly involved in the water drive response and initial histological survey of our prolactin tests shows no thyrotropic or gonadotropic activity. The lactogenic hormone is not effective in promoting molt in hypophysectomized animals as it was in normal efts studied by Chadwick and Jackson (1948). However, as molting did occur in efts receiving injections of TSH it suggests that the increased molting reported by Chadwick and Jackson (1948) in intact animals was due to the endogenous release of TSH resulting from treatment.

Though our investigations are in general agreement with those of Chadwick, we cannot support his assumption that prolactin effects the entire water drive syndrome. Work on hypophysectomized animals indicates that the problem is considerably more complex and can tentatively be divided into four major steps.

1. Migration to water and change of skin texture: induced by prolactin.
2. Normal molting which facilitates but does not directly affect water drive: release of thyroid hormone mediated through the pituitary (TSH).
3. Appearance of olive pigmentation: unknown principle involved, possibly MSH.
4. Morphological characteristics associated with water drive such as keeling of the tail and development of the lateral line system: unknown principle or principles involved.

It is tempting to suggest that prolactin may initiate the entire water drive syndrome by triggering the endogenous release of other endocrines which induce many changes associated with the aquatic phase. The identification of these substances, the parts of the cycle they effect and possible interrelationships involved will be taken up in future papers. In conclusion it appears safe to say that the lactogenic hormone produces water drive and skin change, and that the red eft test for the presence of prolactin (1 I.U. or above) is a positive and reliable one.

SUMMARY

1. Other investigators have shown that the land (eft) stage of *Diemyctylus viridescens* can be induced to enter water and assume adult pigmentation and morphological characteristics following treatment with various pituitary preparations. Hypophysectomized efts were used in the present experiment in order to assure positive identification of the active, water drive principle.

2. Operated animals treated with LH, growth hormone, ACTH, posterior pituitary preparation, TSH, Antuitrin S and melanophore-stimulating hormone gave a negative response. Eight-milligram injections of FSH produced water drive in

several animals, but this was most probably due to the contamination of the substance with prolactin.

3. Most hypophysectomized efts, with the exception of those receiving TSH, either failed to molt or underwent an abnormal molt after the animals had been induced to enter water.

4. Operated animals receiving injections of prolactin (240 to 1.05 I.U.) migrated to water from 4 to 10 days following treatment. However, they failed to acquire adult pigmentation and associated characteristics.

5. The lactogenic hormone has been identified as the principle which initiates the migration of efts to water and the water drive test for prolactin is considered to be reliable.

LITERATURE CITED

Adams, A. E., 1932. Observations on the effect of anterior pituitary extract (phyone) on the 'red phase' of *Triturus viridescens*. *Anat. Rec.*, **52**: 46. (Abst.)

Adams, A. E., and M. C. Grierson, 1932. Cornification and molting in *Triturus*. *Proc. Soc. Exp. Biol. Med.*, **30**: 341–344.

Adams, A. E., A. Kuder and L. Richards, 1932. The endocrine glands and molting in *Triturus viridescens*. *J. Exp. Zool.*, **63**: 1–55.

Chadwick, C. S., 1940a. Induction of water drive in *Triturus viridescens* with anterior pituitary extract. *Proc. Soc. Exp. Biol. Med.*, **43**: 509–511.

Chadwick, C. S., 1940b. The water drive in *Triturus viridescens* as an effect of the growth promoting hormone of the anterior hypophysis. *J. Tenn. Acad. Sci.*, **15**: 412.

Chadwick, C. S., 1940c. Identity of prolactin with water drive factor in *Triturus viridescens*. *Proc. Soc. Exp. Biol. Med.*, **45**: 335–337.

Chadwick, C. S., 1941. Further observations on the water drive in *Triturus viridescens*. II. Induction of the water drive with the lactogenic hormone. *J. Exp. Zool.*, **86**: 175–187.

Chadwick, C. S., 1948. Evidence of a thyroid-skin gland relationship in the induction of molting in the red eft of *Triturus viridescens*. *Anat. Rec.*, **101**: 678. (Abst.)

Chadwick, C. S., and H. R. Jackson, 1948. Acceleration of skin growth and molting in the red eft of *Triturus viridescens* by means of prolactin injections. *Anat. Rec.*, **101**: 718. (Abst.)

Dawson, A. B., 1936. Changes in the lateral line organs during the life of the newt *Triturus viridescens*. A consideration of the endocrine factors involved in the maintenance of differentiation. *J. Exp. Zool.*, **74**: 221–237.

Grant, W. C., and J. A. Grant, 1956. The induction of water drive in the land stage of *Triturus viridescens* following hypophysectomy. *Anat. Rec.*, **125**: 604. (Abst.)

Noble, G. K., 1926. The Long Island newt; a contribution to the life history of *Triturus viridescens*. *Amer. Mus. Nov., No. 228.*

Noble, G. K., 1929. Further observations on the life-history of the newt *Triturus viridescens*. *Amer. Mus. Nov., No. 348.*

Reinke, E. E., and C. S. Chadwick, 1939. Inducing land stage of *Triturus viridescens* to assume water habitat by pituitary implants. *Proc. Soc. Exp. Biol. Med.*, **40**: 691–693.

Reinke, E. E., and C. S. Chadwick, 1940. The origin of water drive in *Triturus viridescens*. I. Induction of the water drive in thyroidectomized and gonadectomized land phases by pituitary implantations. *J. Exp. Zool.*, **83**: 224–233.

Steelman, S. L., W. A. Lamont, W. A. Dittman and E. J. Hawrylewicz, 1953. Fractionation of the swine follicle stimulating hormone. *Proc. Soc. Exp. Biol. Med.*, **82**: 645–647.

Tuchmann-Duplessis, H., 1948. Développement des caractères sexuels du Triton traité par des hormones hypophysaires gonadtropes et lactogènes. *C. R. Soc. Biol.*, **142**: 629–630.

Tuchmann-Duplessis, H., 1949. Action de l'hormone gonadtrope et lactogène sur le comportment et les caractères sexuels secondaires du triton normal et castré. *Arch. Anat. Micr. Morph. Exp.*, **38**: 302–317.

THE SENSITIVITY OF ECHOLOCATION IN BATS

ALAN D. GRINNELL AND DONALD R. GRIFFIN

Biological Laboratories, Harvard University, Cambridge 38, Mass.

The full significance of acoustic orientation in bats can only be understood when we know what kinds of objects are detected and at what distances. Is it true, as is often assumed, that echolocation is limited to very close ranges of a foot or two? To what extent can bats discriminate between different objects? Are they merely aware that something is or is not directly ahead, or does echolocation inform them about the distance, size, numbers, direction and speed of motion of whatever is returning the echoes? Insectivorous bats seem to use echolocation in the pursuit and capture of flying insects; do they distinguish between various kinds of insects? Some continue to hunt insects in the rain; how can they tell the beetles from the raindrops? It would also be helpful to know how the acuity of echolocation varies among the several groups of bats which employ quite different intensities and patterns of sound for echolocation (Möhres, 1953; Griffin and Novick, 1955; Griffin, 1958).

These and related questions call for a better understanding of the sensitivity and effective range of echolocation, and this paper describes some new measurements of the distances at which bats first react to the presence of small wires. Although the smaller species of bats often fly very close to large objects such as the walls of a room before showing any sign of awareness that something is ahead, they do change the pattern of their orientation sounds at somewhat greater distances. For example, a *Myotis lucifugus* commonly increases its pulse repetition rate from perhaps 5 to 10 per second before take-off to 15 or 20 per second during ordinary flight and to 50 or more per second when landing or dodging small obstacles. This increase is closely correlated with success in avoiding objects such as wires. The rate rises every time a normal or blindfolded bat approaches the wires, but deafened bats show no such increase as they fly up to wires which they cannot detect (Galambos and Griffin, 1942). We have utilized this characteristic increase in pulse repetition rate to determine the distance at which bats first react to obstacles of various sizes by thus changing the pattern of their emitted sounds, and the results demonstrate a greater range and sensitivity of echolocation than had previously been recognized.

We are happy to acknowledge our gratitude to the Office of Naval Research for the support of these studies through a contract with Harvard University. Reproduction in whole or in part is permitted for any purpose of the United States Government.

METHODS

Bats were allowed to fly in the rectangular room shown in Figure 1. This room is 10 meters long, 3.7 meters wide, and 2.4 meters high; it was free from furniture or obstructions other than the wires, three observers, and a microphone and camera mounted on tripods. Seven meters from the end of the room, marked A, was a row of vertical wires 30 cm. apart, and 5.5 meters from the same end, in an indentation in one wall, was an Auricon model CM-72 16 mm. sound motion picture camera with a 9.5 mm. lens. In all cases the flying bats stood out against the white background formed by the opposite wall, which was marked only by conspicuously numbered vertical stripes placed at 60-cm. intervals to provide a frame of reference. In each flight used in the present measurements, the bat was released close to point A and flew approximately straight towards the opposite wall, B, passing the wires 7 meters away from its starting point. Usually it turned in the remaining 3 meters or else landed on the end wall.

The observer who released the bat at point A noted its approximate distance from the wall opposite the camera as it flew towards and past the wires. A second observer turned the camera to follow the bat during its flight, which in a typical case approximated the dashed line of Figure 1. The third observer kept the microphone pointed towards the flying bat. The motion pictures showed the flight path of the bat as it appeared from the position of the camera silhouetted against the opposite wall, but a parallax correction (based on the first observer's notes of the bat's distance from the white wall) was necessary except when the animal was directly opposite the camera. The camera was so placed that any errors introduced by parallax were minimized in the region where the pulse repetition rate was beginning to change.

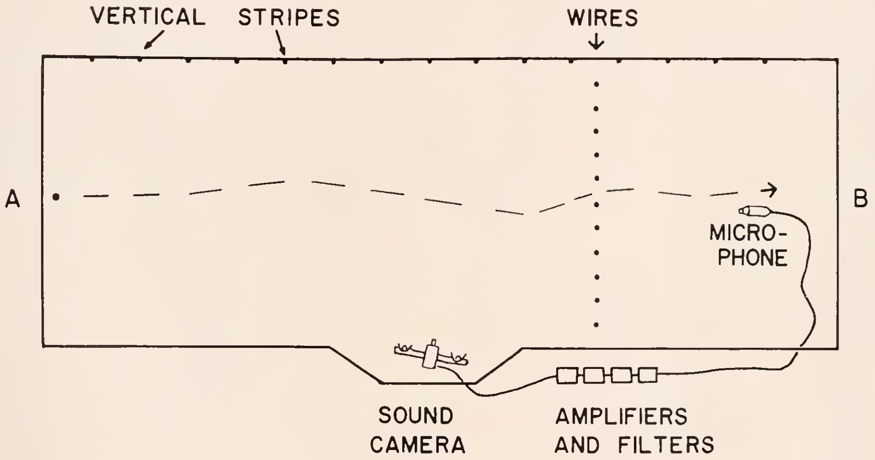


FIGURE 1. Diagram of room used for measurements of the distance of vocal reaction to the wires.

The high frequency sounds emitted by the bat as it flew the length of the room were picked up by a 640AA Western Electric condenser microphone placed 2.1 meters beyond the wires. The amplifiers and associated apparatus were similar to those used in previous studies of bat sounds (Griffin, 1950, 1953). The frequency band from 30 to 80 kc was selected by Spencer-Kennedy Laboratories model 301 and 302 variable electronic filters, 54 db/octave slope at 30 kc high pass and 18 db/octave slope at 80 kc low pass. The amplified signal was then passed through a detecting circuit (the pulse detector used by Griffin and Novick, 1955), and the resulting clicks were recorded directly on the sound track of the same film containing the photographs. The developed film was studied with a time-motion study projector, the single frames being projected one at a time while the corresponding portion of the sound track was moved past a celluloid time scale calibrated in milliseconds. It was thus possible to measure directly for every pulse the position of the bat and the elapsed time since the last pulse.

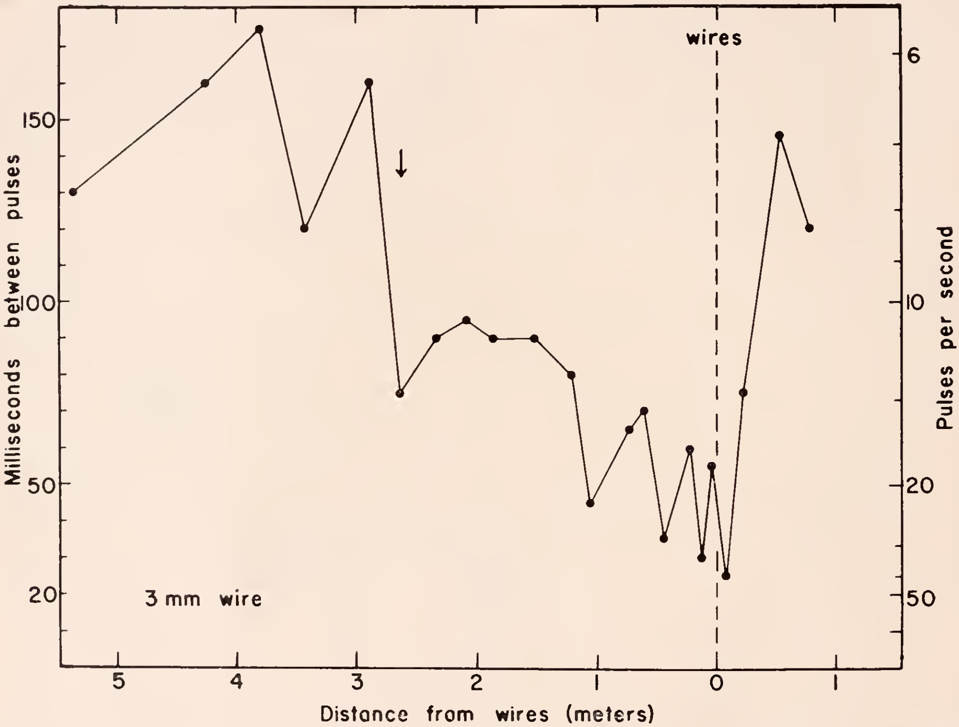


FIGURE 2. Variation of the pulse-to-pulse interval during one flight of a *Myotis lucifugus* through a row of 3-mm. wires along approximately the flight path shown in Figure 1. The arrow indicates the distance of the first vocal reaction.

The bats used in these experiments were *Myotis lucifugus* which had been in captivity for no more than one day, and all were in excellent condition. Six sizes of wire were used: 3 mm., 1.07 mm., 0.65 mm., 0.46 mm., 0.28 mm. and 0.18 mm. in diameter. The 3-mm. wire was rubber-covered, but all the others were bare iron or copper. Out of about 650 flights photographed, 146 were selected for analysis because the bats did react to the wires as demonstrated by straight flights through the wires, usually with a clear effort to dodge them. For this reason there was a larger proportion of misses than would otherwise have been the case. Flights with appreciable turns and flights near the walls, ceiling, or floor were excluded since a close approach to any object is likely to involve a change in pulse repetition rate. We also excluded flights in which the record of the pulses on the sound track was of low amplitude or was complicated by noise, so that there was a danger that some of the pulses might be overlooked in studying the record. Other flights were excluded because the pulse repetition rate varied widely during the 3 to 4 meters of straight flight from point A to the vicinity of the wires or did not return to approximately the same level after the bat passed through the wires. Nor were any flights used unless we were confident that the photographs established the bat's position with an accuracy of ± 10–15 cm. over at least the major part of its flight through the wires. The time required for sound to travel from bat to microphone was only about 0.03 second at the very most, and it decreased gradually as the animal flew towards the wires. Hence the acoustic delay had no appreciable effect on the measurement of the interval between pulses.

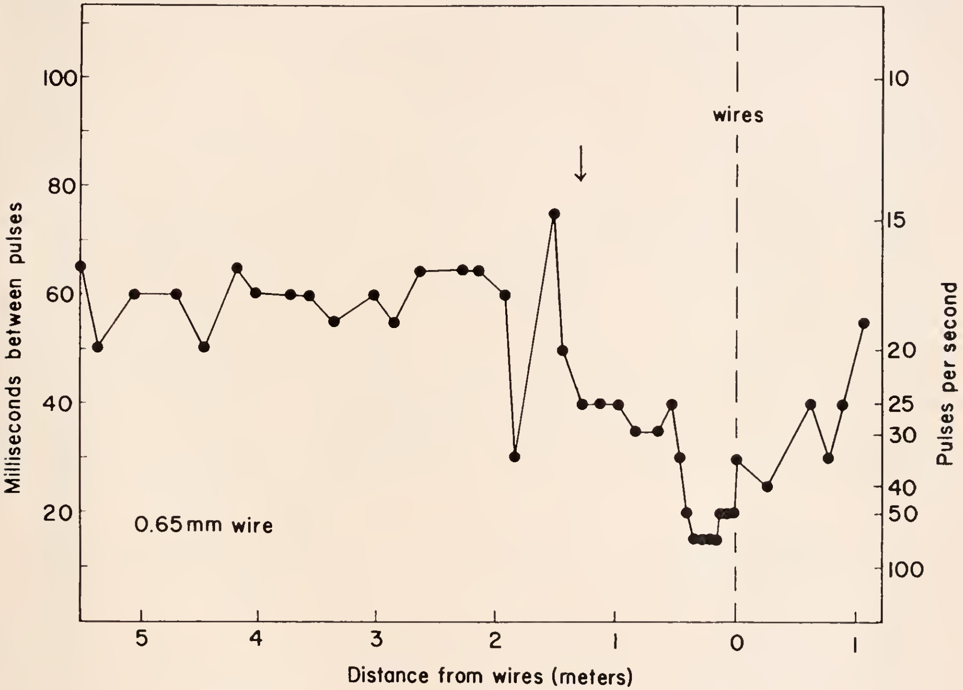


FIGURE 3. Intervals between pulses emitted by a bat flying through a row of 0.65-mm. wires.

RESULTS

More than 500 flights through the wires showed the characteristic increase in pulse repetition rate with only two or three exceptions, all of which occurred with the 0.18-mm. wire. For the 146 flights selected for analysis the bat's position was determined at the time each pulse was emitted, and the pattern of sound emission in typical flights is shown graphically in Figures 2–6, where each point represents a single pulse. Since the repetition rate varies rapidly it is more appropriate to consider the data in terms of the time interval between pulses. Therefore the ordinate of these graphs shows the time elapsed since the previous pulse, together with the corresponding repetition rate. Figures 2–6 show typical examples of these curves with five of the six sizes of wire studied, including cases when the pulse-to-pulse interval was both relatively constant (Fig. 6) or rather variable (Fig. 3) before the approach to the wires, cases in which the actual values of the interval were high (Fig. 2) or low (Fig. 3), and cases in which the drop was slight (Fig. 5) as well as others in which it was very marked (Fig. 4).

In the present experiments the wires were hung from small screw hooks in the ceiling, but the vocal reactions occurred when the bats were flying more than a meter below the ceiling, and thus were most unlikely to be reacting to the hooks. Furthermore, there were many similar hooks elsewhere on the ceiling which caused no change in the emitted sounds, and control tests with no wires hanging from the hooks showed no significant variations in the pulse-to-pulse interval. Each of fourteen bats for which clear records of the first flights are available yielded a typical curve like those shown in Figures 2–6 the first time it flew through the experimental room, showing that the change in pulse repetition pattern was not merely the result of familiarity with the position of the wires.

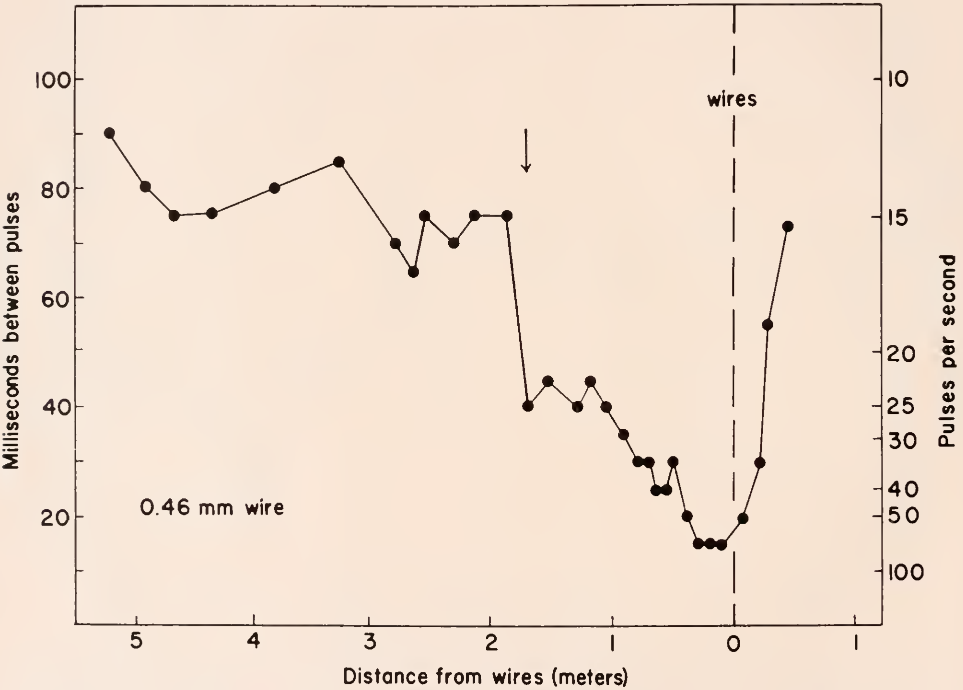


FIGURE 4. Intervals between pulses emitted by a bat flying through a row of 0.46-mm. wires.

The actual values of the pulse-to-pulse interval varied considerably. With a few individuals it was as high as 150–170 msec during the straight flight towards the wires (Fig. 2), with others it was approximately constant at 40 to 60 msec (Fig. 3). Just at the wires the interval sometimes fell to about 10 msec, but in

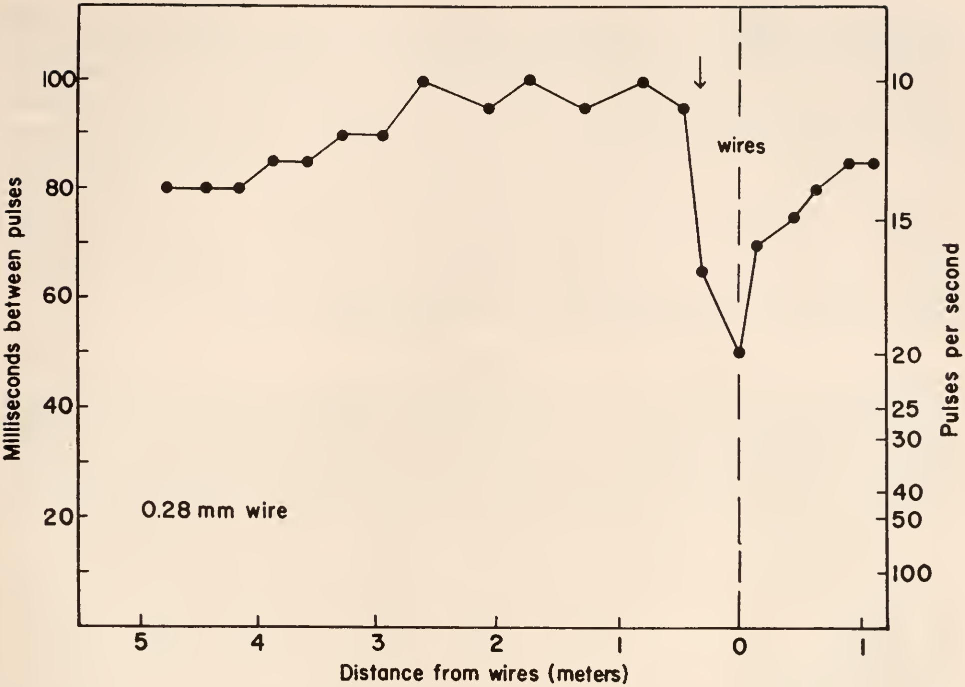


FIGURE 5. Intervals between pulses emitted by a bat flying through a row of 0.28-mm. wires.

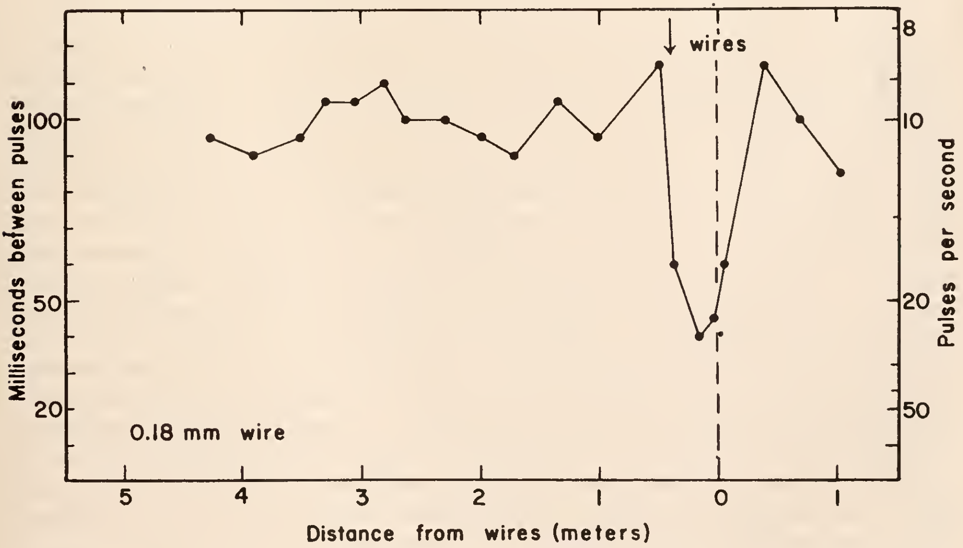


FIGURE 6. Intervals between pulses emitted by a bat flying through a row of 0.18-mm. wires.

other cases, especially with the smaller sizes, it fell only to 40 or 50 msec. Two methods are available for estimating the distance from the wires at which a first vocal reaction occurs with sufficient regularity to be significant. The first is to judge for each curve the approximate point at which the interval first fell significantly below the previous level and the level to which it returned after the wires had been passed. Such estimates could be made with some confidence within ± 15–20 cm., and examples are shown by small arrows on Figures 2–6. The average, minimum and maximum values of such estimates for each of the six sizes of wire are listed in Table I. It was encouraging to obtain nearly the same average distances of first reaction in completely independent series of photographs with the same wires taken several months apart and using different bats.

TABLE I

Distance from wires of various sizes at which Myotis lucifugus first reacted by decreasing the interval between pulses. The wires used were vertical and spaced 30 cm. apart. The individual estimates of the distance of detection were made from curves similar to Figures 2–6; and average, minimum, maximum values of such estimates are shown below. Owing to the uncertainty of such estimates their average is lower than the distance of first reaction to the wires obtained from Figure 7

Diameter of wire (mm.)	3.0	1.07	0.65	0.46	0.28	0.18
No. of bats	10	17	6	6	3	3
No. of flights	29	42	21	17	13	19
Per cent misses	93%	97%	76%	82%	77%	74%
Average est. distance of detection (cm.)	186	144	133	118	92	66
Range	(66–294)	(49–197)	(66–245)	(69–180)	(30–138)	(30–117)
Distance of detection obtained from Fig. 7 (cm.)	215	185	150	120	105	90

A second, and probably better, method is to average the values of the pulse-to-pulse interval measured at various distances from the wires. The results of this type of averaging are shown in Figure 7, together with arrows to indicate the distances at which these average curves first showed a definite drop below the level characteristic of flight before and after approach to the wires. As shown in Table I these estimates of the average distance of first reaction are somewhat greater than those obtained by the first method, presumably because variation due to other factors than proximity to the wires was cancelled out to some extent in the averaging process. Therefore the values obtained from Figure 7 provide the best available measure of the distance at which alert and successful bats of this species first react to wires of these six sizes. Consideration of Figure 7 is somewhat complicated, however, by the fact that not all of the individual curves covered the same range of distances from the wires. Hence the ends of the average curves are based on a smaller number of flights than is listed in Table I. Careful examination of the individual curves did not disclose any significant effects on the average curve of this change in number of flights, and in those more important portions of the average curves that are shown in Figure 7 by solid dots, 90% or more of the number of flights listed in Table I were included in the averages.

Table I shows that with all six sizes of wire there was a large proportion of misses, the remaining flights being "touches" or "hits" as defined by Griffin and Galambos (1941). Study of the few individual curves for touches or hits in the present series showed no appreciable difference from those ending in a miss. This is not inconsistent with the observation of Galambos and Griffin (1942) that there was less likely to be a change in rate in flights ending in a hit, because the present series was initially selected to include only straight flights by bats that were registering a high degree of success at dodging the wires.

DISCUSSION

These measurements are an extension of earlier experiments in which the pulse repetition rate was shown to increase as bats approached wires that were about 1.2 millimeters in diameter. It is therefore important to point out certain differences in the methods used and in some aspects of the results obtained. The apparatus available for the earlier studies was not capable of reliably registering bat pulses at a sufficient distance to provide information such as that presented in Figures 2–7, even if the bat's distance from the wires had been recorded. Furthermore the room was smaller (4.5 meters long instead of 10 meters in the present experiments), and the flights studied were not limited to straight approaches by bats at their optimal level of skill at echolocation. This is probably why a higher proportion of the present series were misses, and why almost every one of the present trials showed a clear decrease in pulse-to-pulse interval as the bat approached the wires. More sensitive apparatus might well have revealed a larger proportion of cases with a slight but detectable change in repetition rate, had it been available in 1941. But this does not alter the fact, demonstrated at that time, that successful bats are much more likely to show a marked change in repetition rate on approaching small obstacles than are those which collide with the wires.

In the original experiments it was our impression from visual observation that the bats ordinarily reverted to a distinctly lower pulse repetition rate just *before* passing through the wires. It is therefore of interest to examine the more extensive and accurate data obtained in the present experiments with respect to the positions at which the pulse-to-pulse interval rose again to approximately the value measured before the bat approached within two meters of the wires. It is clear from the individual flights illustrated in Figures 2–6, as well as from the average curve for each size of wire, that the interval did not completely return to its earlier level until some distance past the wires. In several individual curves, however, the pulse-to-pulse interval appears to have risen shortly before the wires were passed, as in Figure 3. But many other individual curves, such as Figures 2 and 4, show that the pulse-to-pulse interval did not rise appreciably until the wires had been passed. It should be recalled in this connection that the position of the bat was determined only within ± 10–15 cm., and in a majority of individual curves the increase in interval occurred within this distance of the wires. In a substantial minority of cases the first definite increase appeared to be delayed until the bat was more than 15 cm. past the wires (8 out of 29 flights with 3-mm. wire, 17 out of 42 with 1.07-mm., 8 out of 21 with 0.65-mm., 4 out of 13 with 0.46-mm., but only one out of 13 with the 0.28-mm. and 3 out of 19 flights with the 0.18-mm. wire). In only one case, with the 0.28-mm. wire, the curve began to rise more than 15 cm. before the plane of the wires.

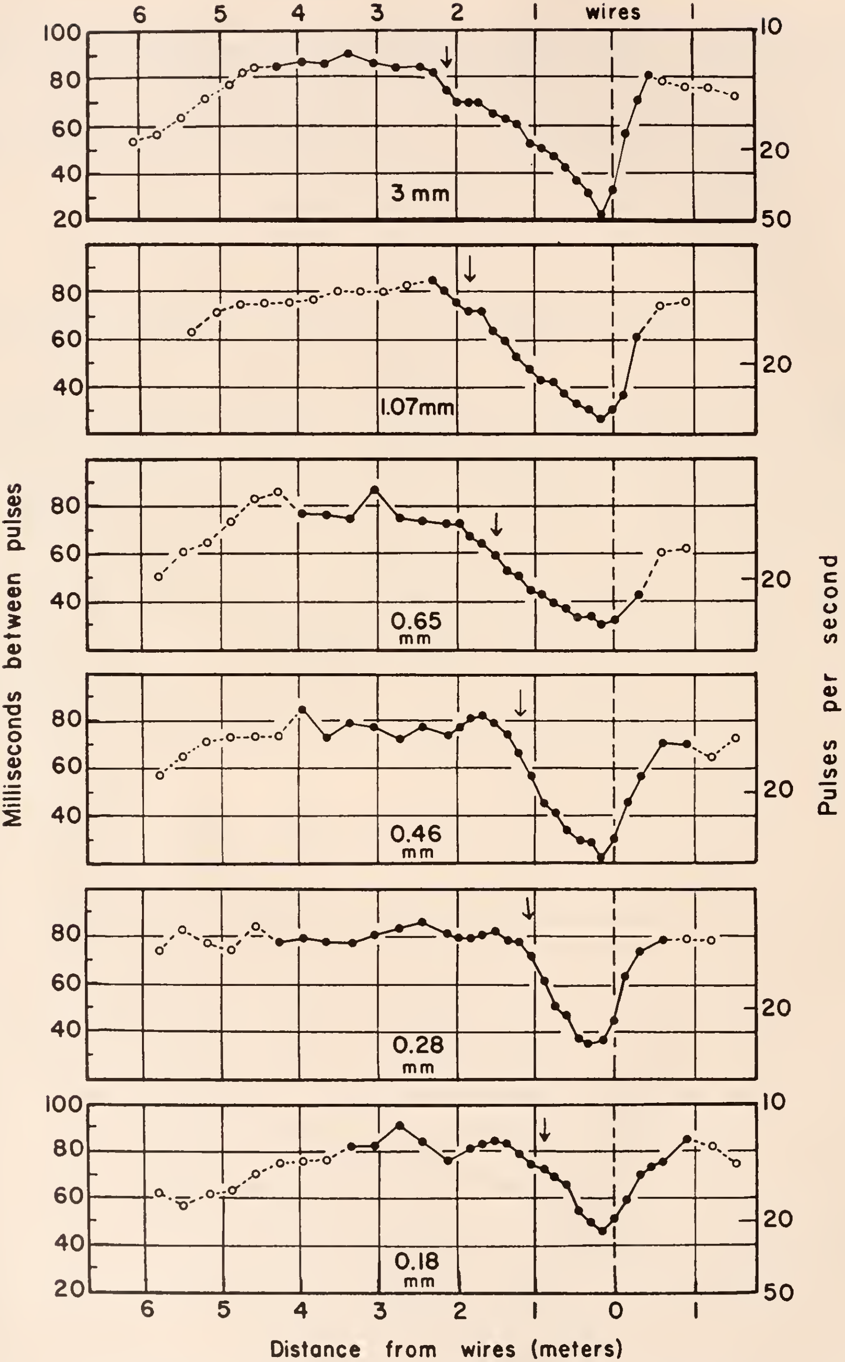


FIGURE 7. Average pulse-to-pulse intervals of bats flying past wires ranging from 0.18 to 3 mm. in diameter. The arrows indicate the distance of first vocal reaction.

The average curves of Figure 7 show a slight increase in the interval just as the bats flew past the wires. But since the possible error of the determinations of the bat's position was ± 10–15 cm. this small difference is only barely significant. In short, these measurements demonstrate only that the rise in the interval between pulses occurred on the average within 15 cm. of the wires, and was apparently more likely to begin shortly before passage through the plane of the wires than shortly after. Yet the 1.07-mm. wire was approximately the same size as the wires used in the earlier tests, and the spacing of the wires was the same. It is not clear whether the difference between our strong impression from observing the first experiments and the results of these more accurate measurements resulted from the selection of more alert and skillful bats for the present series, from the larger size of the room, or from some other factor.

In many flights the pulse-to-pulse interval just before the bat reached the wires alternated somewhat regularly between two quite different values, as in Figure 2. In other words the pulses tended to come in pairs, each pair separated from the next by an interval somewhat greater than that between the members of each pair. A similar tendency for pulses to occur in groups of two, three or occasionally four, was apparent in the first graphic records of bats' orientation pulses (Galambos and Griffin, 1942). Perhaps these groups correspond to the respiratory cycle. In the present series of curves the presence of this feature is clearly correlated with the size of the wires themselves, as shown in Table II.

TABLE II

Number of flights showing a marked alternation in the interval between pulses, similar to that illustrated in Figure 2

Diameter of wire (mm.)	Definite alternation		No alternation		Doubtful	
	Number	Per cent	Number	Per cent	Number	Per cent
3.0	25	86%	2	7%	2	7%
1.07	23	55%	11	26%	8	19%
0.65	12	57%	4	19%	5	24%
0.46	5	26%	10	53%	2	11%
0.28	1	8%	10	77%	2	15%
0.18	2	10.5%	15	79%	2	10.5%

Whatever significance this alternation may have, it was more likely to occur with the larger sizes of wire. Perhaps there was simply not time during the last quarter of a second or so of flight up to the 0.18- or 0.28-mm. wire for so complicated a vocal reaction. Indeed, if the decrease in pulse-to-pulse interval did not occur until the bat was closer than one meter to the wires, the period of increased repetition rate often contained only five or six pulses. Whatever additional information the bat obtained from the extra pulses over and above those that would have been produced at the previous rate, its vocal reaction was a brief and limited one.

The pattern of sound emission has been discussed above in terms of time, but it is also of interest to consider it in terms of space. The same photographs show how fast the bats were moving towards and past the wires, and the average of 54 velocity measurements was 3.9 meters per second, with the extremes of the series 2.4 and 6.3 meters/second. The speed did not vary significantly with the different sizes of wire, nor at different portions of the individual flights, except in a few cases when a late turn to dodge a wire caused momentary slowing and fluttering. Since the interval between pulses averaged about 70 milliseconds before the repetition rate had increased in proximity to the wires, a flight velocity of four meters per second means that one pulse was emitted about every 28 cm. As the bats flew within 0.5 meter of the wires the interval often fell to 20–30 msec, and the lower figure corresponds to one pulse every 8 cm. of flight at 4 meters/sec., or approximately once every time the bat moved through a distance equal to its own length. When the flight slowed in front of the wires, even shorter distances separated the positions at which successive pulses were emitted.

The actual detection of an echo from the wires must of course have preceded the first vocal reaction of the bat, and hence the distance of detection was somewhat greater than the distance of reaction discussed above. To consider the 3-mm. wire, for example, the average distance of reaction was 215 cm. But the first pulse to occur after a shortened interval was not registered until it had travelled to the microphone located 210 cm. beyond the wires, or 425 cm. from the bat. The acoustic delay for this distance is about 13 msec. A further correction should be made for the bat's reaction time, which might have been as little as 15 msec (the approximate time required for the contraction of the intra-aural muscles at the onset of a loud sound), or perhaps it was as long as 200 msec (the order of magnitude of minimum human reaction times). A conservative estimate of 25 msec for the sum of acoustic delay and reaction time indicates that when a bat detected the echoes of the 3-mm. wires it was at least 10 cm. farther from the wires than our data demonstrate directly. A similar estimate for the 0.18-mm. wires places the bat about 100 cm. when their echoes first became audible. If this does not appear to be a very impressive range of detection it is well to bear in mind that it is about 5500 times the diameter of the wires themselves.

The success of bats in avoiding wires naturally varies with the diameter of the wire; but even when wires are spaced 30 cm. apart the percentage of misses of an alert *Myotis lucifugus* does not fall sharply until the diameter of the wire is reduced below about 0.3 mm. In a long series of experiments with this species performed by Curtis (1952) in a smaller flight room, the average percentage of misses of wires spaced the same distance apart varied as follows with the wire diameter: 4.8 mm., 85%, 1.21 mm., 85%, 0.68 mm., 77%, 0.35 mm., 72%, 0.26 mm., 52%, 0.12 mm., 39%, and 0.07 mm., 36%. The chance score at this spacing is about 35%. These bats had been less highly selected for skillful flight and obstacle avoidance than those in the present series. For example, the three bats tested with 0.18-mm. wire registered 32 misses out of 46 flights photographed. The only selection involved in this series was the decision that the bat was flying well and tending to head straight towards the wires so that it was worthwhile to take pictures of it.

In view of the small size of the wires, relative to the wave-lengths of the emitted sounds, it is surprising that the distance of detection did not vary more with wire size. While the ratio of wire diameters was about 17:1, the distances of reaction varied only by less than 2.5:1. If Raleigh scattering was the chief source of the echoes, the ratio of echo intensities at a fixed distance should have been $(17)^4$:1

(Morse, 1948). To be sure, the echo intensity also varies inversely as the cube of the distance (since the echo radiates from wires as a series of cylindrical waves), and atmospheric attenuation reduces the echo somewhat further. Even if we assume the echo intensity to vary inversely as the fourth power of the distance, we still face a puzzling discrepancy of $(17)^4/(2.5)^4$ or more than two thousand.

One way to escape from this dilemma is to postulate that much higher frequencies or shorter wave-lengths are used to detect these small wires, perhaps harmonics of the fundamental frequencies in the bat's orientation pulse. This might bring the wire diameters up to the order of one wave-length so that Raleigh scattering would not predominate, and the echo intensity would vary more slowly with wire diameter. But all available evidence indicates that the maximum intensity of the emitted pulse, and the maximum sensitivity of hearing, both occur at about 50–60 kc, or a wave-length of 6 to 7 mm., where all but the two larger sizes should be within the range of Raleigh scattering. At higher frequencies the echo intensity and the sensitivity of hearing would probably both fall off fairly rapidly.

Another and perhaps better explanation would be that the bats could actually detect the wires at greater distances than our data indicate, but that they do not trouble themselves to increase the pulse repetition rate until they come within a meter or two. The relatively small increase in distance of vocal reaction between the 1.07- and 3-mm. wire could be explained about equally well by assuming that the echo strength increased less rapidly as the wire diameter approached one wave-length, or by postulating that the 3-mm. wire was detected at a greater distance but did not elicit a vocal reaction until about two meters. We cannot resolve this question without new and more refined experimental evidence. It is interesting to note in this connection a suggestion of a double break in the curves for the 1.07- and 3-mm. wires. It is possible that a slight reduction in the pulse-to-pulse interval occurs somewhat earlier than the onset of the pronounced drop which is apparent at all six wire sizes. About one-third of the individual curves for the 3-mm. wire have a distinct double break, as shown, for example, in Figure 2.

Since these insectivorous bats apparently detect small wires at 1.0 to 2.25 meters it is natural to inquire whether larger objects can be located at correspondingly greater distances. One factor which limits a simple extrapolation to larger sizes and longer ranges of detection is the attenuation of high frequency sound in air. (For values of the coefficient of atmospheric absorption in the bats' frequency range see Beranek, 1949, pages 64–72.) Furthermore the intensity of the echo falls off as approximately the third or fourth power of the distance, depending upon the geometry of the object reflecting or scattering the sound. Nevertheless it must be possible for these bats to detect objects several centimeters in size at considerably greater distances, and really large objects such as trees or buildings are presumably detectable at distances of many meters. No methods have yet been devised, however, to determine objectively the maximum distances at which such objects are first detected by bats, and this fact presents a real challenge to future students of echolocation and bat behavior.

SUMMARY

1. The distance at which bats (*Myotis lucifugus*) react to the presence of a row of small wires has been measured by a photographic determination of the

distance at which the pulse repetition rate first increases as the bats fly towards the wires. Distinct changes in this rate were measured in almost every flight towards wires spaced 30 cm. apart and ranging in diameter from 0.18 to 3 mm.

2. The interval between successive pulses averaged 60 to 80 msec as the bats flew along the room towards the row of wires, and dropped to 20–40 msec just before the barrier. The intervals decreased less with the smaller sizes of wire.

3. All but the largest of these wires are well below one wave-length of the emitted sounds of these bats (50–60 kc, or 6–7 mm., at the peak intensity and 120 kc, or about 3 mm., at the very beginning of some pulses).

4. Clear evidence that the wires had been detected was furnished at the point where the interval between pulses first dropped significantly below the level that prevailed before and after the approach to the row of wires. This average distance of first vocal reaction to the row of wires was 215 cm. for 3-mm. wire, 185 cm. for 1.07-mm., 150 cm. for 0.65-mm., 120 for 0.54-mm., 105 cm. for 0.28-mm., and 90 cm. for 0.18-mm. A conservative correction for reaction time and the acoustic delay between the bat and the microphone indicates that the distance of first detection must have been at least 10 cm. greater than these distances of reaction.

5. Since small wires can be detected at distances of as much as 5500 times the wire diameter, and well before the bat gives evidence by its flight pattern that it is aware of them, it appears likely that larger objects are detected at considerably greater distances.

LITERATURE CITED

Beranek, L. L., 1949. Acoustic measurements. John Wiley and Sons, New York.

Curtis, W. E., 1952. Quantitative studies of echolocation and vision in bats and owls. Thesis deposited in Library of Cornell University, Ithaca, N. Y.

Galambos, R., and D. R. Griffin, 1942. Obstacle avoidance by flying bats; the cries of bats. *J. Exp. Zool.,* **89**: 475–490.

Griffin, D. R., 1950. Measurements of the ultrasonic cries of bats. *J. Acoust. Soc. Amer.,* **22**: 247–255.

Griffin, D. R., 1953. Bat sounds under natural conditions, with evidence for the echolocation of insect prey. *J. Exp. Zool.,* **123**: 435–466.

Griffin, D. R., 1958. Listening in the dark. Yale University Press, New Haven, Conn.

Griffin, D. R., and R. Galambos, 1941. The sensory basis of obstacle avoidance by flying bats. *J. Exp. Zool.,* **86**: 481–506.

Griffin, D. R., and A. Novick, 1955. Acoustic orientation of neotropical bats. *J. Exp. Zool.,* **130**: 251–300.

Möhres, F. P., 1953. Über die Ultraschallorientierung der Hufeisennasen (Chiroptera-Rhinolophidae). *Zeitschr. f. vergl. Physiol.,* **34**: 547–588.

Morse, P. M., 1948. Vibration and sound. McGraw Hill Book Co., New York. Sec. Edit.

PHYSIOLOGY OF INSECT DIAPAUSE. XI. CYANIDE-SENSITIVITY OF THE HEARTBEAT OF THE CECROPIA SILKWORM, WITH SPECIAL REFERENCE TO THE ANAEROBIC CAPACITY OF THE HEART[1]

WILLIAM R. HARVEY[2] AND CARROLL M. WILLIAMS

The Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

Among the metabolic changes which accompany the onset of insect diapause is a pronounced decrease in sensitivity to cyanide and carbon monoxide. This fact was first discovered by Bodine and Boell (1934a, 1934b) in diapausing eggs of the grasshopper *Melanoplus,* and has subsequently been studied in further detail in *Melanoplus* (Robbie *et al.,* 1938; Robbie, 1941) and in the Cecropia silkworm. The situation in the case of Cecropia may be summarized as follows.

Cyanide and carbon monoxide are lethal agents for the caterpillar of the Cecropia silkworm—a fact which mirrors the presence in the larval insect of an intact and functional cytochrome system. However, immediately after pupation the cytochrome system undergoes partial breakdown in all tissues except the intersegmental muscles of the abdomen. Simultaneously, the over-all metabolism of the diapausing pupa becomes substantially insensitive to cyanide and high pressures of carbon monoxide. This state of affairs persists throughout the prolonged period of pupal diapause. Finally, months later, the termination of diapause and initiation of adult development are accompanied by re-synthesis of cytochromes and the appearance of a fresh increment of metabolism which is sensitive to carbon monoxide. If one blocks this increment with cyanide or carbon monoxide, the insect's development is brought to a standstill.

On the basis of these findings one may infer that the metabolism during the growing, non-diapausing stages in the life history is mediated by the usual cyanide- and carbon monoxide-sensitive cytochrome oxidase. In this sense there is nothing remarkable about the insect's metabolism before and after the pupal diapause. But what is remarkable is the character of the metabolism of the diapausing insect itself. The clear-cut resistance to cyanide and carbon monoxide suggests that the metabolism of the diapausing insect proceeds *via* some simpler and more primordial system of electron transfer making use of a terminal oxidase other than cytochrome oxidase. Under this point of view, the metabolism of the diapausing pupa is conceived to differ, not only quantitatively, but also qualitatively, from that before and after diapause. This prospect has been examined experimentally by Schneiderman and Williams (1954a, 1954b) and incorporated into a comprehensive theory of the biochemistry of diapause.

Crucial to this interpretation is the breakdown of the cytochrome system at

---

[1] This investigation was supported, in part, by a grant from the National Cancer Institute of the U. S. Public Health Service.

[2] Predoctoral Fellow of the Public Health Service and the Lalor Foundation.

the outset of pupal diapause—a matter which has recently been re-examined by Shappirio and Williams (1957a, 1957b) in individual tissues of the Cecropia silkworm. Spectrophotometric studies at room temperature and spectroscopic studies at the temperature of liquid nitrogen confirm that, in all tissues except the intersegmental muscles of the abdomen, rapid changes in the cytochrome system take place at the outset of pupal diapause. Within 24 hours after the pupal molt, cytochromes *b* and *c* decrease at least 30-fold and become indetectable; cytochrome b_5 and cytochrome oxidase ($a + a_3$) likewise decrease at this same time, but then stabilize at low but finite levels. The net effect is that throughout the pupal diapause the tissues contain cytochrome oxidase in large excess over cytochrome *c*. Consequently, if the cyanide- and carbon monoxide-sensitive system fails to participate in the metabolism of the diapausing tissues, then the block in electron transfer must be localized at the level of cytochrome *c* rather than at the level of cytochrome oxidase itself.

Whether cytochrome *c* actually disappears at the outset of diapause is a matter which lies beyond the resolution of the most sensititive methods of assay available at the present time. This is a question of decisive importance because a low concentration of *c* in the presence of a tremendous excess of oxidase might camouflage the participation of the cytochrome oxidase system in the metabolism of diapause. Thus, by means of carbon monoxide or cyanide one could poison, say, 95 per cent of cytochrome oxidase activity and the residual 5 per cent of active oxidase might still be able to saturate cytochrome *c* and sustain the low and "carbon monoxide-insensitive" metabolism of the diapausing insect.

Because of the limitation inherent in methods for the assay of cytochrome *c*, the problem appeared to be intractable to further biochemical analysis at the present time. Therefore, we have directed attention back to the insect itself. We have selected for intensive study the physiology of a particular tissue, the insect heart. Through an investigation of this tissue we have been able to bypass many of the above-mentioned difficulties and to comprehend what appears to be the mechanism of cyanide and carbon monoxide-sensitivity and -insensitivity in the Cecropia silkworm. In the present paper attention is directed to the effects of cyanide on the heartbeat of the insect during metamorphosis. In the following paper (Harvey and Williams, 1958) the effects of carbon monoxide will be considered.

MATERIALS AND METHODS

1. *Experimental animals*

The experimental animals, *Platysamia cecropia* L., were reared and managed according to methods described previously (Williams, 1946, 1956). Experiments were performed on the insect at the following stages: mature larvae shortly before the initiation of spinning; unchilled pupae which had been stored at 25° C.; chilled pupae which had been stored at 6° C.; chilled pupae which had been stored for 4 to 6 months at 6° C. and then returned to 25° C. for one week; post-diapausing individuals at successive stages in adult development at 25° C.; and adult moths which had developed and emerged at 25° C. Certain experiments were performed in parallel on the related Polyphemus silkworm (*Telea polyphemus* Cram.).

2. *Methods*

A. *Exposure of isolated hearts to increasing concentrations of cyanide*

The dorsal half of the abdomen was excised with scissors and pinned by its lateral margins to a wax layer in the bottom of a circular dish of Lucite (polymethyl methacrylate). Each dish was provided with a Lucite cover and with inlet and outlet tubes arranged in such a manner that the preparation was automatically bathed in 20 ml. of gently flowing insect Ringer's solution (Ephrussi and Beadle, 1936). The latter was slightly modified by the substitution of 0.001 *M* potassium phosphate buffer (pH 7.0) for a corresponding proportion of the potassium chloride. To the solution prior to use were added a few milligrams of a 1:1 mixture of crystalline phenylthiourea and streptomycin sulfate—the former to block tyrosinase activity and the latter to oppose bacterial growth.

The gut and gonads were removed from the preparation, thereby exposing the heart and alary muscles. The paired masses of fat body were pressed aside so that the heart could be viewed *in situ* through a dissecting microscope.

The physiological solution was aerated continuously by a gentle stream of oxygen introduced into the fluid by a 20-gauge hypodermic needle passing through the lateral wall of the dish. A 26-gauge needle passing through the plastic cover permitted the addition of a solution of hydrogen cyanide; a reservoir of the latter was stored in a one-liter Pyrex wash bottle which was connected by Tygon tubing to the hypodermic needle.

The preparation was first equilibrated with insect Ringer until the heartbeat was stabilized. This ordinarily required one to two hours. The flow of Ringer was stopped and the cyanide solution was then dripped into the perfusion fluid at a rate of approximately ten drops per minute. The concentration of cyanide in this stock solution was 10 to 100 times the inhibitory level, as ascertained in preliminary experiments. The dropwise addition of cyanide was continued until the heartbeat was strongly inhibited. The oxygen flow was shut off and a two-ml. sample of the perfusion fluid was then immediately withdrawn into a hypodermic syringe and analyzed for cyanide by the phenolphthalin technique described by Robbie (1944).

B. *Exposure of isolated hearts in a flowing system*

An elongate plastic tube, 1.9 cm. in outside diameter, was cut longitudinally to form two semi-cylindrical troughs. The depression was then filled with melted wax. A series of hearts was isolated and pinned to the wax bottom of the plastic trough; the latter was then slipped into a glass tube (60 cm. long and I. D. 2 cm.). The glass tube was equipped with ground glass joints at its two ends. One end was connected by the ground joint to a stoppered reservoir containing the solution to be tested. The latter was forced from the reservoir by a slight positive pressure of overlying oxygen or nitrogen. The solution, after flowing slowly over the abdomens, made exit from the ground joint at the distal end of the glass tube and was passed in rubber tubing into a five-gallon bottle containing strong alkali. As the occasion required, samples of solution were withdrawn from the rubber tube with a hypodermic syringe and analyzed for cyanide or for oxygen.

C. *Appraisal of heartbeat*

In Method A the constant agitation of the oxygen bubbles caused considerable irregularity in the frequency of beat. Therefore, in appraising the heartbeat in experiments utilizing Method A, primary attention was centered on the amplitude of the beat rather than its frequency. This method of study was soon abandoned in favor of Method B. Here the frequency of heartbeat was found to be far more constant and predictable. Under constant conditions the variability of heart rate was small compared to that brought about by the experimental treatment. Routinely the frequency of heartbeat was counted for each individual over a period of from one to five minutes and averaged as beats per minute. The strength (amplitude) of the beat was also scored as normal (3), subnormal (2), barely detectable (1), and absent (0). In order to obtain an over-all index of heart function, the recorded frequencies were divided by 1 when the heartbeat was normal, by 2 when the beat was subnormal, and by 3 when the beat was barely detectable. We shall hereafter refer to this value as the "heartbeat index."

D. *Reagents*

Cyanide was obtained as potassium cyanide (Mallinckrodt) assaying not less than 96.0%. Fresh solutions were prepared daily in oxygenated Ringer, neutralized with 1 N hydrochloric acid to pH 7.0, and stored in stoppered containers in the cold. At this pH, 98% of the cyanide is present as hydrocyanic acid.

The experimental gases were obtained in pressure cylinders and assayed as follows: "pre-purified nitrogen" (Airco), 99.998%; oxygen (Airco), 99.5%.

RESULTS

1. *Acute poisoning of the isolated heart*

Isolated hearts of Cecropia, at successive stages in metamorphosis, were exposed during a period of one-half hour to increasing concentrations of cyanide by Method A. The concentration required to inhibit the heartbeat during this half hour was ascertained for each of a series of animals at each of seven stages in metamorphosis. When judged in this manner, the cyanide-sensitivity of the heartbeat is found to undergo large and systematic changes during the course of metamorphosis.

As recorded in Table I and Figure 1, the heartbeat of the mature larva is blocked within 0.5 hour by cyanide concentrations somewhat less than 10^{-3} *M*. However, immediately after the pupal molt, a remarkable resistance to cyanide becomes evident. Thus, within one day after the molt, the inhibitory cyanide concentration increases to 5×10^{-3} *M*. This trend continues until, some two to three weeks later, the inhibitory concentration is not far short of 10^{-1} *M*. The net effect is that the transition of the larva into a diapausing pupa is accompanied by a 100-fold decrease in sensitivity to acute poisoning by cyanide. This condition then persists during the months of pupal diapause.

After prolonged exposure to 6° C., the pupal diapause is terminated; only one or two days at 25° C. are then required for the visible initiation of adult development (Williams, 1956). Though pupae of this type show no detectable development when examined immediately after their return to 25° C., it is of interest that resistance to cyanide has already begun to decline (Table I and Fig. 1). By the first or second day of adult development the heart is approximately as sensitive to cyanide as the larval heart. During the three-week period of adult development at 25° C., one records an ever-increasing sensitivity to acute poisoning by cyanide. Finally, the heart of the freshly emerged adult moth is blocked by cyanide at concentrations as low as 10^{-5} M—a sensitivity 8,000 times that recorded for the diapausing pupa.

TABLE I

Acute toxicity of cyanide for Cecropia and Polyphemus hearts: cyanide concentrations which block the heartbeat during 0.5-hour exposure*

P. cecropia

Stage	No. of preparations	Final concentration of cyanide ($\times 10^{-4}$ M)	Reversibility of effects
Fifth instar larva	7	7.5 ± 0.46**	+
One-day-old pupa	6	50.0 ± 4.80	0
Pupa after 2–3 weeks at 25° C.	6	770.0 ± 170.00	0
Pupa after 8 months at 6° C.	12	77.0 ± 6.90	0
First or second day of adult development at 25° C.	5	5.1 ± 1.20	+
Fifteenth or sixteenth day of adult development at 25° C.	6	3.0 ± 1.70	+
Adult moth	21	0.1 ± 0.04	+

T. polyphemus

Stage	No. of preparations	Final concentration of cyanide ($\times 10^{-4}$ M)	Reversibility of effects
Pupa after 5 months at 25° C.	6	350.0 ± 44.00	0
Pupa after 7 months at 6° C.	11	31.0 ± 8.30	0
Eleventh or twelfth day of adult development at 25° C.	4	12.0 ± 3.10	0
Adult moth	8	0.5 ± 0.21	+

* No beat or only trace of beat during one minute of observation.
** Standard error.

As summarized in Table I, the observations were repeated on pupae and adults of the Polyphemus silkworm (*Telea polyphemus*). Here again, the cyanide resistance of the pupal heart is evident.

For both these species the response of the pupal hearts to acute poisoning by cyanide is remarkable, not only in terms of the high concentrations required to inhibit the heartbeat, but also in terms of the irreversibility of this inhibition (Table I). Whereas the heartbeat of larvae, developing adults, and adults is promptly re-

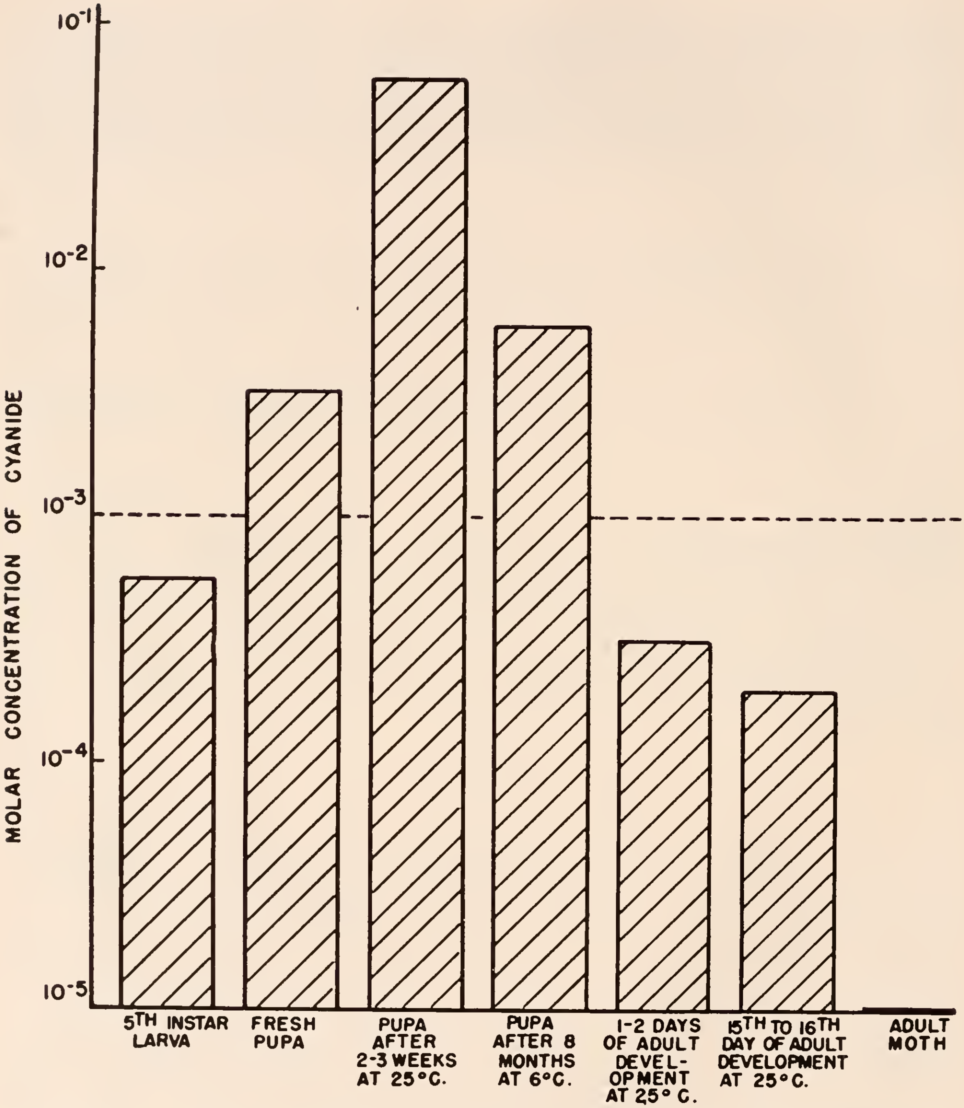


Figure 1. Cyanide concentrations required to block the beat of the isolated heart of the Cecropia silkworm within 0.5 hour. The resistance of the heart to acute cyanide-poisoning is seen to undergo major changes during the larval-pupal-adult transformation.

gained when returned to cyanide-free Ringer, the pupal hearts are evidently killed by the high concentrations required to inhibit them.

The experiment therefore directs attention to the paradoxical behavior of the pupal heart in relation to poisoning by cyanide. As illustrated in Figure 1, the pupal heart continues to beat normally for at least a half hour in cyanide concentrations far exceeding 10^{-3} *M*; that is, under conditions where one would anticipate the inhibition of the vast majority of cytochrome oxidase activity. How can one account for this resistance of the pupal heart to cyanide?

One possibility is that the pupal heart contains a terminal oxidase other than cytochrome oxidase, and that this unknown oxidase is insensitive to cyanide. However, it was necessary to consider an even simpler explanation; namely, that the pupal heartbeat can be sustained by strictly anaerobic processes.

2. *Pupal heartbeat under anaerobic conditions*

The hearts of four diapausing pupae were isolated and pinned in the bottom of the glass tube described under Method B above. The tube was first perfused with a gently flowing stream of oxygenated Ringer, and the heartbeat of each individual ascertained. The 400-ml. tube was then perfused rapidly with oxygen-free insect Ringer; the perfusion was then continued at the lower rate of approximately 500 ml. per hour. Special attention was given to the total removal of oxygen from the physiological solution prior to its use. For this purpose, pre-purified nitrogen was bubbled through the Ringer for at least two hours; moreover, after traversing the solution the nitrogen was bubbled through a solution of reduced methylene blue (Fildes, 1931). The absence of color change gave assurance that all oxygen had been removed from the Ringer. The latter was then stored under a slight positive pressure of pre-purified nitrogen, and displaced by this pressure through the tube containing the hearts.

The hearts of four diapausing pupae were studied—first in air, then for 5½ hours in oxygen-free Ringer, and, finally, for 43 hours in oxygenated Ringer. The various measurements are summarized in Table II, along with the average heartbeat indices.

One is immediately impressed by the striking resistance of these diapausing hearts to strictly anaerobic conditions. After 0.5 hour of anaerobiosis, none of the hearts showed any detectable depression. After one hour, only one of the four was depressed. Between the first and second hours the over-all index value decreased

TABLE II

Effects of strictly anaerobic conditions on isolated hearts of brainless diapausing Cecropia pupae

Animal No.	Rate (beats/min.) and amplitude* of heartbeat										
	Air	Hours in Ringer equilibrated with pre-purified nitrogen						Subsequent hours in oxygenated Ringer			
		½	1	2	3	4½	5½	¼	1½	25	43
1	12 (3)	17 (3)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	8 (3)	22 (3)	17 (3)	10 (3)
2	13 (3)	13 (3)	12 (3)	12 (2)	13 (3)	5 (3)	4 (2)	14 (3)	17 (3)	13 (3)	9 (3)
3	17 (3)	19 (3)	19 (3)	13 (2)	0 (0)	14 (2)	0 (0)	20 (3)	4 (3)	6 (3)	7 (3)
4	12 (3)	20 (3)	19 (3)	14 (3)	12 (2)	8 (1)	0 (0)	26 (3)	19 (3)	13 (3)	8 (3)
Average heartbeat index	13.5	17.2	12.5	6.8	4.8	3.8	0.5	17.0	15.5	12.2	8.5

* See Methods.

markedly; however, one of the hearts showed a normal beat after two hours of anaerobiosis and another, after three hours. Three of the four hearts were still beating after 4½ hours of anaerobiosis, and one, after 5½ hours.

It will be observed in Table II that the effects of 5½ hours of anaerobiosis were promptly reversed when the hearts were returned to oxygenated Ringer. Indeed, the average index values actually increased for about an hour over the level at the outset, and then stabilized at or near the initial, normal level. The absence of any permanent damage attributable to anaerobiosis is also confirmed by the continuation of heartbeat for 1½ further days until the experiment was abandoned.

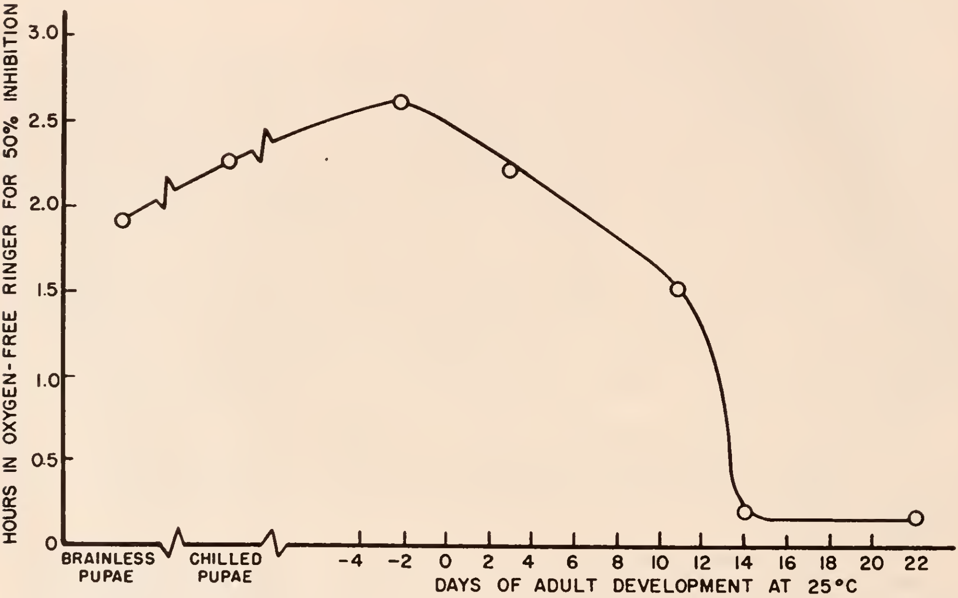


FIGURE 2. The "anaerobic reserve" of the isolated Cecropia heart is plotted as a function of pupal-adult development. The discontinuities in the curve correspond to days or weeks of storage under the conditions noted on the X-axis. The anaerobic reserve declines almost to zero during the course of adult development. Each datum is the average derived from the hearts of four to eleven individuals.

3. *Heartbeat of chilled pupae, developing adults, and adults under anaerobic conditions*

Is the high degree of facultative anaerobism peculiar to the heart of the diapausing pupa? In order to answer this question the experiment, just considered, was repeated on the hearts of: previously chilled pupae; chilled pupae that had been returned to 25° C. and were just prior to the initiation of adult development; developing adults; and adults. The results are recorded in Figure 2 in terms of the period of anaerobiosis required for the reversible inhibition of 50 per cent of the average heartbeat index. The method of arriving at this value is illustrated in Figure 3. The average heartbeat indices of the diapausing pupae are here plotted as a function of the duration of exposure to oxygen-free Ringer. A smooth curve is drawn by inspection through the series of points and the time for 50 per cent inhibition is ascertained from the curve.

In the results summarized in Figure 2, it is clear that a considerable capacity for anaerobism persists within the pupa during the months of chilling at 6° C. When pupae of this type are placed at room temperature, the capacity for anaerobism actually appears to increase slightly. By the fourth day of adult development the "anaerobic capacity" has returned to the level observed in diapausing pupae. This trend continues and by the eleventh day of adult development the time for 50 per cent inhibition under anaerobic conditions has dropped to 1.5 hours. On or about the eleventh day of adult development the anaerobic capacity decreases precipitously to the low level characteristic of the adult moth. The adult moth, emerging after 21 days of development at 25° C., is maximally sensitive to oxygen lack in that the heart is able to beat less than 10 minutes in the total absence of oxygen.

In the experiments just considered, the anaerobic condition was established by the use of oxygen-free Ringer's solution. The observations on pupal and adult hearts were repeated in a series of experiments in which the anaerobic condition was established by the ventilation of the tube with a flowing stream of pre-purified nitrogen gas (300 ml. per hour). Precisely the same results were observed.

In a further series of experiments making use of pre-purified nitrogen, the findings were confirmed in studies of the pupal and adult hearts of *Telea polyphemus.*

Consequently, for both these species, it is clear that the pupal heart, unlike the adult heart, possesses a substantial "anaerobic reserve" which can sustain the beating of the heart for as long as 5½ hours in the total absence of oxygen. Aside from the intrinsic interest of this new finding, the anaerobic capacity of the pupal heart is obviously critical in the design of experiments testing the aerobic metabolism of the pupal heart.

4. *Sensitivity of the pupal heart to prolonged exposure to cyanide*

In Section 1 the pupal heart was found to be extremely resistant to cyanide. However, it will be recalled that this result was based on experiments of short duration in which the heart was exposed to increasing concentrations of cyanide during a period of 0.5 hour. We now see that the pupal heart can beat for up to 5½ hours in the total absence of oxygen. The earlier experiments were therefore inadequate as a test of the cyanide sensitivity of the pupal heart. For this reason the effects of cyanide on the pupal heartbeat were re-examined in experiments of prolonged duration.

Isolated pupal hearts were placed in the flow tube (Method B) and subjected to a flowing stream of oxygenated insect Ringer containing a precise concentration of cyanide. The reservoir of Ringer was prepared in a stoppered five-gallon bottle and stored under oxygen. In order to cause the Ringer to flow through the experimental tube, the reservoir was slightly compressed by the addition of a stream of oxygen; the latter was bubbled through an aqueous solution of 10^{-1} or 10^{-2} M potassium cyanide before entering the reservoir. In this manner it was possible to prevent any significant change in the cyanide concentration in the Ringer during prolonged experiments. This fact was confirmed by cyanide assays performed on fluid that had traversed the chamber.

Cyanide at two specific final concentrations was studied in detail; namely, 10^{-2} *M* and 10^{-3} *M*. It will be recalled that both these concentrations were without detectable effects on the pupal heartbeat in experiments of short duration.

The effects of 10^{-2} *M* cyanide are summarized in Figure 4. In terms of the average index values, the heartbeat remained normal for 1½ hours. Two of the six hearts stopped beating after 2 hours. After 3½ hours, all hearts showed considerable depression, and three of the six had stopped. The average time required for 50 per cent inhibition was 2.25 hours. The tube was then flushed with cyanide-free Ringer. Three hearts showed a slight recovery at this time. This was a temporary effect, however, for all six hearts were in standstill after a total of two hours in cyanide-free Ringer.

In like manner the effects of perfusion with oxygenated Ringer containing 10^{-3} *M* cyanide were studied. A considerable depression was first evident after 2¾ hours, but all four hearts were still beating after 4 hours. At the end of 5¼ hours, two of the hearts stopped beating and the other two showed only a trace of beat. At this time the system was flushed with cyanide-free Ringer. All four hearts showed a delayed recovery and three of the four were beating normally after a total of 16 hours in cyanide-free Ringer.

Discussion

The heartbeat of the larva and the adult Cecropia silkworm is blocked in a reversible manner by brief exposure to cyanide in concentrations less than thousandth molar. Therefore, on the basis of this classical test, it seems safe to conclude that the hearts of the larva and the adult moth make use of cytochrome oxidase as "terminal oxidase."

When the same criterion is applied to the pupal heart, the latter is found to beat normally when immersed in cyanide at concentrations not far short of tenth molar. Here, then, is a tissue which appears to be totally insensitive to cyanide over the range of concentrations at which cyanide is an inhibitor of cytochrome oxidase. Consequently, the pupal heart has appeared to be a clear instance of a cyanide-insensitive tissue whose function is not dependent on metabolism mediated by cytochrome oxidase. Prior to the present investigation, we have routinely thought that this was so (Williams, 1951; Harvey and Williams, 1953).

On the basis of the experimental results described above, it is now clear that the pupal heart is by no means insensitive to cyanide. The crux of the matter is that the true cyanide-sensitivity of the heart is camouflaged most effectively by an anaerobic capacity peculiar to the pupal heart. Whereas the adult heart is able to beat for less than ten minutes in the total absence of oxygen, the pupal heart, by contrast, beats normally for one or more hours under the same conditions.

The experimental conditions leave little room for doubt that the pupal heartbeat, during this prolonged period of facultative anaerobism, is sustained by strictly anaerobic metabolism. Under this circumstance the heart can scarcely require the function of cytochrome oxidase or, for that matter, any other enzyme concerned with the utilization of atmospheric oxygen. Therefore, for a corresponding period the pupal heart is found to be totally insensitive to physiological concentrations of cyanide.

The true sensitivity of the pupal heart to cyanide is unmasked only when one continues the experiment sufficiently long to use up the anaerobic reserve. This fact is evident in a comparison of Figures 3 and 4. The inhibition of the pupal heart by cyanide (Fig. 4) shows precisely the same time-course as that observed under anaerobic conditions in the absence of cyanide (Fig. 3). As the heart exhausts its anaerobic reserve, it becomes progressively more dependent on aerobic metabolism and progressively more sensitive to cyanide. These observations strongly argue that the aerobic metabolism of the diapausing heart requires the presence and function of cytochrome oxidase.

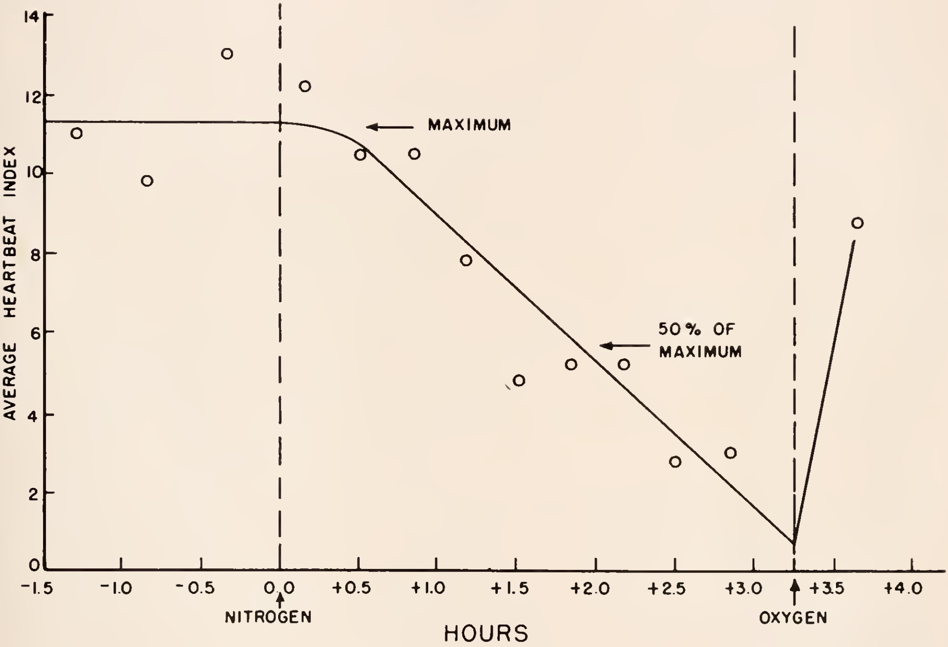


FIGURE 3. Technique for determining the time required for the 50 per cent inhibition of the heartbeat index during exposure of isolated hearts to oxygen-free Ringer. Each datum is the average from the hearts of eight brainless diapausing pupae.

On the basis of our present data we are unable to state the lower limit of cyanide concentration which inhibits the pupal heart in experiments of this type. However, for reasons which will be considered in detail in the following paper, we doubt that the pupal heart can ever be inhibited by the very low cyanide concentrations (10^{-5} M) which suffice to block the heartbeat of the adult moth.

While clarifying the problem of the cyanide-insensitivity of the pupal heart, the present study directs attention to a fresh problem—the changes occurring in the insect's capacity for anaerobic metabolism during the course of metamorphosis. As illustrated in Figure 2, these changes are large and systematic. Of particular interest is the rapid loss of "anaerobic reserve" which supervenes approximately midway in adult development.

We suspect that this change is not peculiar to the heart. Thus, according to Schneiderman and Williams (1954b), mature larvae and adult moths of the Cecropia silkworm are killed in less than one day when exposed to "tank nitrogen" containing less than 0.5% oxygen. Diapausing pupae, by contrast, survive more prolonged exposures, the L.D. 50 per cent being three days.

On the basis of present inadequate information we are unable to comprehend the full meaning of this shift in the capacity for anaerobic metabolism. The quantitative changes suggested in Figure 2 are almost precisely the reverse of those occurring in the over-all aerobic metabolism and in the concentration of such typically aerobic enzymes as the cytochromes. The problem obviously merits further study.

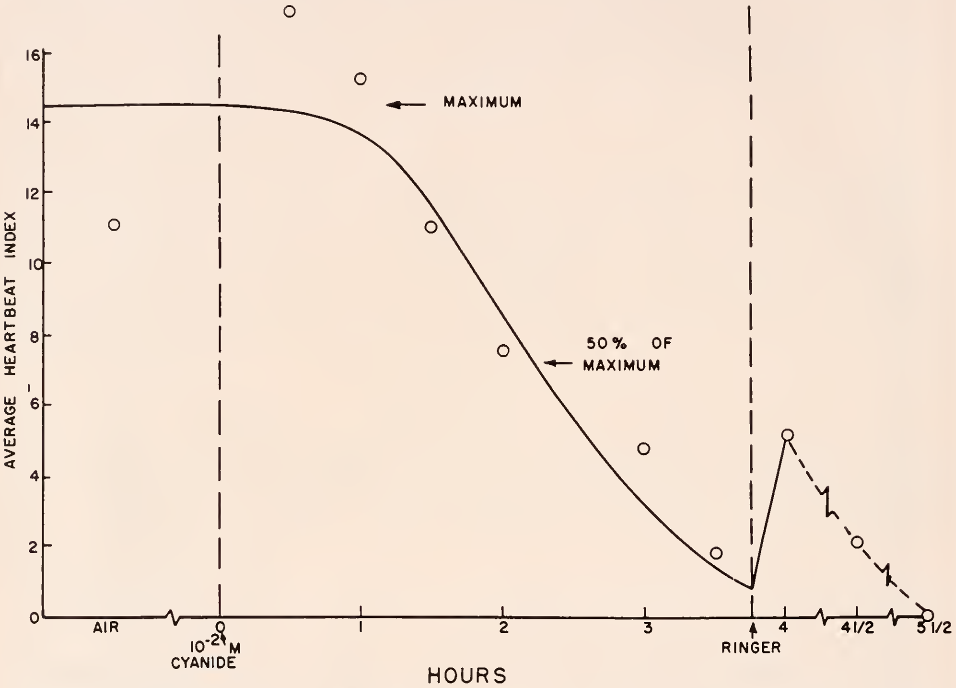


FIGURE 4. Effects of 0.01 M cyanide on the beat of hearts isolated from diapausing Cecropia pupae. Each datum is the average derived from the hearts of six brainless diapausing pupae.

SUMMARY

1. During the course of metamorphosis the heart of the Cecropia silkworm appears to undergo pronounced shifts in its sensitivity to cyanide.

2. In the mature larva the heartbeat is promptly blocked by 10^{-3} M cyanide; in the adult moth it is even more sensitive and is brought to a standstill by 10^{-5} M cyanide.

3. In the intervening pupal stage the heart is insensitive to acute poisoning by physiological concentrations of cyanide.

4. This insensitivity is observed only in experiments of short duration. When the exposure to cyanide is continued for many hours, the pupal heartbeat is blocked by 10^{-2} or 10^{-3} M cyanide.

5. The paradoxical response of the pupal heart can be accounted for in terms of a pronounced capacity for anaerobic metabolism which is peculiar to this particular stage. The pupal heart can beat for as long as 5½ hours in the complete absence of oxygen. During this same period the heart is insensitive to cyanide.

6. While discounting any true insensitivity of the pupal heart to cyanide, the experimental results direct attention to major and previously unsuspected changes in the anaerobic capacity of the Cecropia silkworm during the course of metamorphosis.

LITERATURE CITED

Bodine, J. H., and E. J. Boell, 1934a. Carbon monoxide and respiration: action of carbon monoxide on the respiration of normal and blocked embryonic cells (Orthoptera). *J. Cell. Comp. Physiol.*, **4**: 475–482.

Bodine, J. H., and E. J. Boell, 1934b. Respiratory mechanisms of normally developing and blocked embryonic cells (Orthoptera). *J. Cell. Comp. Physiol.*, **5**: 97–113.

Ephrussi, B., and G. W. Beadle, 1936. A technique of transplantation for *Drosophila*. *Amer. Nat.*, **52**: 218–225.

Fildes, P., 1931. Anaerobic cultivation. Chapter VI in System of Bacteriology, **9** (Gt. Brit.) Medical Research Council: 92–99.

Harvey, W. R., and C. M. Williams, 1953. Changes in the cyanide sensitivity of the heartbeat of the Cecropia silkworm during the course of metamorphosis. *Anat. Rec.*, **117**: 544.

Harvey, W. R., and C. M. Williams, 1958. Physiology of insect diapause. XII. The mechanism of carbon monoxide-sensitivity and -insensitivity during the pupal diapause of the Cecropia silkworm. *Biol. Bull.*, **114**: 36–53.

Robbie, W. A., 1941. The action of cyanide on eggs and embryos of the grasshopper, *Melanoplus differentialis*. *J. Cell. Comp. Physiol.*, **17**: 369–384.

Robbie, W. A., 1944. An improved phenolphthalin technique for the microdetermination of cyanide. *Arch. Biochem.*, **5**: 49–58.

Robbie, W. A., E. J. Boell and J. H. Bodine, 1938. A study of the mechanism of cyanide inhibition: I. Effect of concentration on the egg of *Melanoplus differentialis*. *Physiol. Zoöl.*, **11**: 54–62.

Schneiderman, H. A., and C. M. Williams, 1954a. The physiology of insect diapause. VIII. Qualitative changes in the metabolism of the Cecropia silkworm during diapause and development. *Biol. Bull.*, **106**: 210–229.

Schneiderman, H. A., and C. M. Williams, 1954b. The physiology of insect diapause. IX. The cytochrome oxidase system in relation to the diapause and development of the Cecropia silkworm. *Biol. Bull.*, **106**: 238–252.

Shappirio, D. G., and C. M. Williams, 1957a. The cytochrome system of the Cecropia silkworm. I. Spectroscopic studies of individual tissues. *Proc. Roy. Soc. London, Ser. B*, **147**: 218–232.

Shappirio, D. G., and C. M. Williams, 1957b. The cytochrome system of the Cecropia silkworm. II. Spectrophotometric studies of oxidative enzyme systems in the wing epithelium. *Proc. Roy. Soc. London, Ser. B*, **147**: 233–246.

Williams, C. M., 1946. Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull.*, **90**: 234–243.

Williams, C. M., 1951. Biochemical mechanisms in insect growth and metamorphosis. *Fed. Proc.*, **10**: 546–552.

Williams, C. M., 1956. Physiology of insect diapause. X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the Cecropia silkworm. *Biol. Bull.*, **110**: 201–218.

PHYSIOLOGY OF INSECT DIAPAUSE. XII. THE MECHANISM OF CARBON MONOXIDE–SENSITIVITY AND –INSENSITIVITY DURING THE PUPAL DIAPAUSE OF THE CECROPIA SILKWORM[1]

WILLIAM R. HARVEY[2] AND CARROLL M. WILLIAMS

The Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

In the preceding paper of this series (Harvey and Williams, 1958), the effects of cyanide on the heartbeat of the Cecropia silkworm were studied during successive stages in metamorphosis. When suitable allowance was made for the anaerobic capacity of the diapausing pupa, the heartbeat at all stages was found to be sensitive to cyanide.

This finding, in itself, suggests that the heartbeat throughout the life history depends on the function of cytochrome oxidase. However, it will be recalled that cyanide combines with a number of enzymes in addition to cytochrome oxidase (Warburg, 1949). For this reason we have re-examined the matter making use of a far more specific inhibitor, carbon monoxide. In animals lacking hemoglobin, a light-reversible inhibition by carbon monoxide is sufficient proof of the presence and functional activity of cytochrome oxidase (Hill and Hartree, 1953).

MATERIALS AND METHODS

1. *Experimental animals*

Diapausing pupae and adult moths of the giant silkworm, *Platysamia cecropia* L., were used as experimental animals. The diapausing individuals were of two types: pupae stored at 25° C. and utilized within three months after the pupal molt; and pupae stabilized in a permanent diapause by removal of the brain at least one month prior to use (Williams, 1946). In a number of cases, parallel experiments were performed on the related giant silkworm, *Telea polyphemus* Cram.

2. *Observations of hearts*

Due to surface reflection from the pupal cuticle, the heartbeat is not visible in the intact insect. Hearts were therefore studied after isolation as previously described (Harvey and Williams, 1958). Subsequently, in collaboration with Dr. Ned Feder, we found that the heart becomes plainly visible and can be studied in the intact pupa under the following circumstance. The light source is equipped with a polarizing filter. A second polarizing filter is placed below the objective of the dissecting microscope. When the planes of polarization are "crossed," surface

---

[1] This investigation was supported, in part, by a grant from the National Cancer Institute of the U. S. Public Health Service.

[2] Predoctoral Fellow of the Public Health Service and the Lalor Foundation.

reflection is totally eliminated. Except in very darkly pigmented individuals one can then clearly observe the beating heart.

3. *Exposure of hearts to gases at elevated pressures*

A series of from four to six hearts was isolated and pinned to an elongate paraffin-coated tray of Lucite. Ringer's solution (Ephrussi and Beadle, 1936), containing phenylthiourea and streptomycin sulfate, was then added until each heart was covered by a film of the solution. In experiments with intact pupae the insects were placed dorsal side up on a Lucite tray equipped with plastic cradles to accommodate the individual pupae.

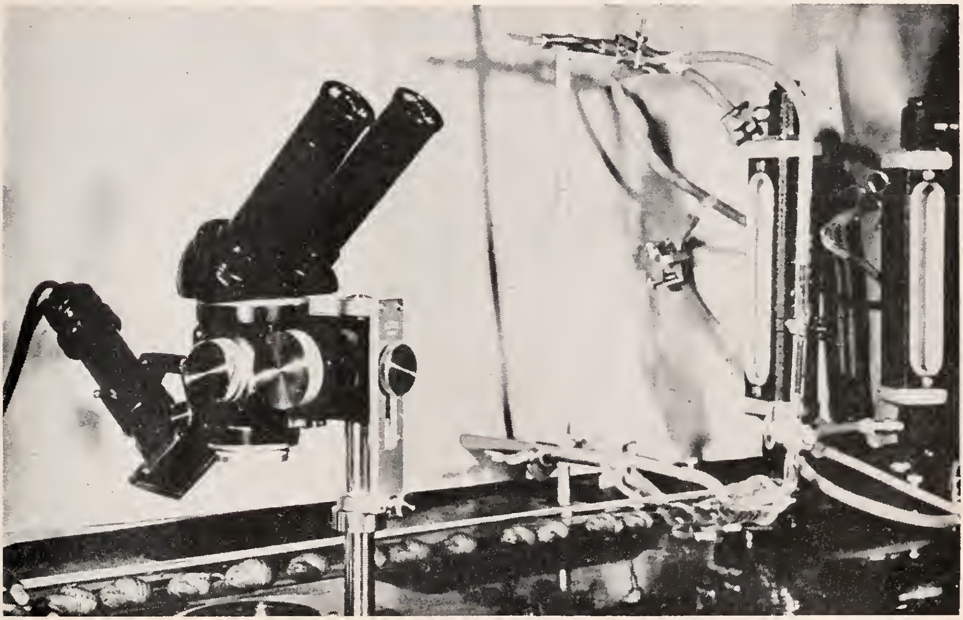


FIGURE 1. Apparatus for the exposure of pupae or of isolated hearts to flowing gas mixtures.

The tray with either isolated hearts or intact pupae was then inserted into a 3.5-liter Lucite chamber constructed to withstand high pressure (Schneiderman and Feder, 1954). The air-filled chamber was then sealed and compressed with carbon monoxide, the positive pressure being read from a gauge calibrated in pounds per square inch. The oxygen tension was therefore that of the initial air-filled chamber (21% atm.). In experiments utilizing oxygen pressures less than 21% atm., the chamber was first flushed with ten volumes of carbon monoxide. The system was then compressed with oxygen to a pre-determined pressure, making use of a mercury manometer. Finally, the chamber was further compressed with carbon monoxide.

4. *Exposure of hearts to flowing mixtures of gases*

As shown in Figure 1, intact pupae were placed in linear sequence in the depression of an elongate, semi-cylindrical tray of cellulose acetate and slipped into the glass tube described under Method B by Harvey and Williams (1958). In experiments utilizing isolated hearts, the latter were pinned to a wax-coated tray and placed in the tube. The proximal end of the flow-tube was connected by a ground joint to a source of a constant flowing gas mixture; the distal end was connected by a ground joint to a length of rubber tubing which passed to a nearby chemical hood.

Specific mixtures of oxygen, nitrogen, carbon monoxide, and air were metered from Rotameters (Tri-Flat Variable-Area Flow Meters, Fisher and Porter, Co.). By selection of meters of appropriate capacity it was possible to utilize the several gases at rates of flow variable from 2 to 1000 cc. per minute, the error of each measurement not exceeding ten per cent. The several gas streams were combined and passed *via* rubber tubing to the experimental chamber, the total gas pressure being one atmosphere in all experiments of this type.

5. *Appraisal of heartbeat*

In experiments utilizing intact pupae, the average frequency and amplitude of the heartbeat were remarkably constant and predictable when computed for a series of individuals. The same was true to a somewhat lesser degree in experiments performed on isolated hearts in the flow-system described above. Therefore, in these types of preparations the average "heartbeat index" (Harvey and Williams, 1958) was used as an over-all expression of the average frequency and amplitude of the heartbeat. The frequency of heartbeat failed to show this same degree of regularity in the case of isolated hearts subjected to pressures greater than one atmosphere. Therefore, in experiments of this latter type, primary attention was centered on the amplitude of the beat rather than on frequency. Amplitude was scored on an arbitrary graded scale from 0 (no beat) to + + + (normal beat).

6. *Illumination*

By virtue of the transparent walls of the experimental chambers, the beating of the isolated heart was visible under the low magnification of the dissecting microscope. Incident illumination was utilized; namely, a focussed 15-watt lamp (Osram H3 6 volts) at a distance of approximately thirteen cm. from the preparation. In experiments pertaining to the light-reversibility of the inhibition, a second lamp (General Electric 1493, 30 watts) was also focussed on the same preparation. The lamps were equipped with one or more filters. An infrared filter (Corning No. 3962), was used routinely. Polarizing filters were used in studies of the intact animal (see above). And in experiments utilizing carbon monoxide mixtures, observations were performed in red light by the use of a Wratten filter (F filter No. 29).

7. *Reagents*

The experimental gases were obtained in compressed cylinders assaying as follows: "pre-purified nitrogen" (Airco), 99.998%; oxygen (Airco), 99.5%; anhydrous compressed air (New England Gas Products). The carbon monoxide was the Matheson product having the following composition: 96.8% carbon monoxide; 0.36% carbon dioxide; 0.97% hydrogen; 1% nitrogen; 0.8% saturated hydrocarbons; 1.19 mg. sulfur per liter. In order to minimize these several impurities, the gas was subdivided by sintered glass filters and bubbled through a succession of two wash bottles containing 10 per cent potassium hydroxide; it then traversed a tube of anhydrous calcium chloride and passed to the flow-meters.

RESULTS

1. *Effects of carbon monoxide on the heartbeat of pupae and adults*

The hearts of seven pupae and twelve adults of the Cecropia silkworm were isolated, moistened with a film of insect Ringer containing streptomycin sulfate and phenylthiourea, and placed in the transparent, high pressure chamber described above under Methods. The strength of the heartbeat was studied in red and in white light in each of three CO/O_2 mixtures; namely, 100/1, 80/1, and 24/1, the oxygen tension in each case being recorded in Table I. Each preparation was observed for two hours.

TABLE I

Effects of carbon monoxide on the heartbeat of diapausing pupae and adult moths of Platysamia cecropia; light-reversibility of carbon monoxide inhibition

CO/O_2 Ratio	Oxygen tension (% atm.)	Amplitude of heartbeat			
		Diapausing pupae		Adult moths	
		Red light	White light	Red light	White light
100/1	5	+++	+++	++	+++
100/1	5	+++	+++	0	+++
100/1	5	++	+++	0	++
100/1	5			0	+++
100/1	5			+	+++
100/1	5			0	+++
100/1	5			0	+++
100/1	5			0	++
100/1	5			0	+++
80/1	5	+++	+++		
80/1	5	+++	+++		
80/1	5	+++	+++		
24/1	20	+++	+++	+	+++
24/1	20			+	+++
24/1	20			++	+++

The results are summarized in Table I. It will be observed that even the highest CO/O_2 ratios had little or no effect on the hearts of the diapausing pupae when the exposure was continued for two hours. By contrast, the adult hearts were strongly inhibited and this inhibition was fully or almost fully reversible in white light.

The light-reversibility in the case of adult hearts was especially dramatic when one region of heart was illuminated with white light and a closely adjacent region, with red light. The resumption of heartbeat in all cases was limited to the region receiving the white light, the adjacent regions within the same heart remaining fully inhibited by carbon monoxide.

The experiments just considered for the Cecropia silkworm were repeated with similar results on adult hearts of the Polyphemus silkworm (*Telea polyphemus*).

The resistance of the pupal heart to high pressures of carbon monoxide was particularly impressive. However, it will be recalled that the heart at this stage possesses a substantial anaerobic metabolism (Harvey and Williams, 1958). Is it possible to account for the resistance to carbon monoxide on this basis? In order to test this possibility a series of four pupal hearts was isolated and exposed to a continuous flowing gas mixture containing 5% atm. oxygen and 95% atm. carbon monoxide (CO/O_2 ratio of 19/1). The carbon monoxide treatment was continued for 20 hours; *i.e.*, for a period greatly in excess of the anaerobic capacity of the pupal heart.

TABLE II

Effects on isolated hearts of prolonged exposure to a gas mixture containing 5 per cent oxygen in carbon monoxide (CO/O_2 ratio = 19/1)

Animal No.	Rate (beats/min.) and amplitude* of heartbeat								
	Air		Hours in CO/O_2 mixture						5 hours in air after 20 hours in CO/O_2 mixture
			2	3¾	5½	7	8½	20	
1	14 (3)	16 (3)	0 (0)	10 (3)	10 (3)	13 (3)	3 (3)	13 (3)	9 (2)
2	6 (3)	9 (3)	7 (3)	9 (3)	11 (3)	3 (3)	3 (3)	2 (3)	0 (0)
3	11 (3)	12 (3)	5 (3)	6 (3)	8 (3)	8 (3)	9 (3)	6 (3)	6 (3)
4	20 (3)	23 (3)	17 (3)	9 (3)	12 (3)	9 (3)	13 (3)	7 (3)	3 (3)
Average heartbeat index**	13	15	7.2	8.5	10	8.2	7.0	7.0	3.5

* Amplitudes scored and recorded in parentheses after rate.

** See Harvey and Williams (1958) for calculation of average heartbeat index.

The results are summarized in Table II. When examined in red light, all hearts were still beating at the end of 20 hours of treatment; indeed, the average heartbeat index was as high at this time as after two hours of exposure to carbon monoxide. Moreover, the performance of the hearts was not improved when they were transferred to air at this time. Therefore, this experiment, like the preceding one, reveals little clear-cut evidence of sensitivity of the pupal heart to carbon monoxide.

2. *The adult heart: further studies on the photo-reversal of CO-inhibition*

A series of five adult hearts was isolated and exposed to a CO/O_2 ratio of 100/1, the oxygen tension being 5% atm. and the carbon monoxide tension 5 atm. Examination under red light confirmed the prompt cessation of heartbeat. In three preparations the effect was fully reversed by white light; in two preparations partially reversed.

The inhibited hearts were exposed, in turn, to a series of five emission lines from a mercury lamp (General Electric, AH5, 200 watts). The various lines were isolated by an appropriate series of Corning filters. Table III records the various combinations of filters and the relative energies of the corresponding emission lines; the latter were determined by the use of a thermopile in conjunction with a U. S. Bureau of Standards source.

In the results recorded in Table III no compensation has been made for the dissimilar relative energies. Notwithstanding this fact, it is sufficiently clear that the 436 and 579 mμ lines were maximally effective in reversing the carbon monoxide effect. These wave-lengths are in good proximity to the 450 and 589 mμ absorption maxima reported for the cytochrome oxidase-carbon monoxide complex of rat heart (Melnick, 1942). Of special interest is the low effectiveness of light at 546 mμ notwithstanding its high relative energy. This emission and those at 365 and 405 mμ fall in regions of minimal absorption reported for the cytochrome oxidase-carbon monoxide complex.

TABLE III

Effectiveness of mercury emission lines in reversing the CO-inhibition of adult Cecropia and Polyphemus hearts*

Species	Wave-length	White	365 mμ	405 mμ	436 mμ	546 mμ	579 mμ
	Relative energy**		0.69	0.49	1.00	4.27	1.71
	Corning filter combination		No. 7380 No. 5860	No. 3060 No. 4308 No. 5970	No. 3389 No. 5113	No. 3484 No. 5120 No. 4303	No. 3480 No. 4305
Cecropia	Amplitude of heartbeat	+++	0	+	+++	+	+++
Cecropia		++	0	0	0	0	+
Cecropia		++	0	+	+++	+	+
Polyphemus		+++	0	0	++	0	+
Polyphemus		+++	0	0	+++	0	+++

* 5% atm. O_2 plus 500% atm. CO (ratio CO/O_2 = 100/1).
** Multiplication by 701 ergs/cm.^{2}/sec. converts to absolute energy.

The action spectrum—rough as it is—leaves little doubt that the effect of carbon monoxide on the adult heart depends on its combination with cytochrome oxidase. Therefore, cytochrome oxidase may confidently be identified as the terminal oxidase of the adult heart. However, the results, up to this point, suggest that cytochrome oxidase may not play this same role in the pupal heart. For this reason the pupal heart was studied in further detail.

3. *Oxygen tension and the pupal heartbeat*

Physiological activities which depend on the function of cytochrome oxidase may be recognized, not only in terms of their light-reversible inhibition by carbon monoxide, but also by the fact that they proceed at normal rates at very low oxygen tensions. This results from the extraordinary affinity of cytochrome oxidase for oxygen. According to Winzler (1941), the oxygen consumption of yeast becomes maximal when the partial pressure of oxygen is only 0.25 to 2.5 mm. Hg. (0.03% to 0.3% atm.)—an affinity for oxygen which far exceeds that of any other known oxidase.

Having failed by the use of carbon monoxide to identify the terminal oxidase in the pupal heart, we sought to clarify the matter by studying the effects of oxygen tension on the function of the pupal heart. For this purpose a series of ten intact diapausing pupae, selected for pale pigmentation, was placed dorsal-side-up in a glass tube. Flowing mixtures of oxygen and nitrogen were then prepared and circulated through the tube, as described above under Section 4 of Methods. The heartbeat was observed within the intact pupae by the use of polarized light.

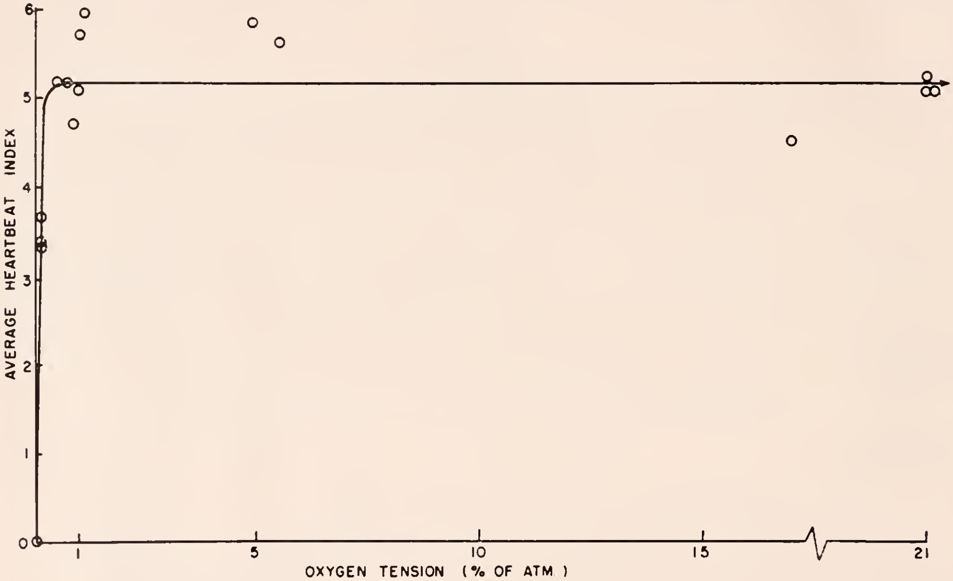


FIGURE 2. Effects of oxygen tension on the heartbeat of intact diapausing pupae of the Cecropia silkworm. The heartbeat is seen to be independent of oxygen at tensions at or above 0.5% atm. oxygen. In the determination of each datum, the exposure was continued for at least eight hours in order to compensate for the "anaerobic reserve."

In order to compensate for the anaerobic capacity of the pupal heart, the pupae were equilibrated with each gas mixture for eight hours prior to scoring the heartbeat. The same ten animals were used for the entire study, the tube being ventilated for eight hours with air before testing the next gas mixture.

The results are summarized and plotted in Figure 2 in terms of the average heartbeat index as a function of oxygen tension. It is extraordinary to observe that the heartbeat was independent of external oxygen tensions at or above 0.5% atm. Only when the pressure of oxygen was reduced below 0.5% atm. (4 mm. Hg) was there any detectable effect.

This result strongly argues that cytochrome oxidase is the terminal oxidase in the pupal heart, since no other oxidase is known to have the high affinity for oxygen implied in Figure 2.

4. *CO-inhibition of the pupal heartbeat at low oxygen tensions*

The finding that the hearts of intact diapausing pupae beat indefinitely at very low oxygen tensions paved the way for a more rigorous study of the effects of carbon monoxide than had been possible heretofore. Hearts could now be exposed to CO/O_2 ratios of 100/1 or higher. Moreover, the effects of carbon monoxide could be studied in the presence of very low oxygen tensions.

The following series of experiments had the objective of studying the heartbeat in the presence of varying pressures of carbon monoxide while holding the oxygen constant at a low pre-determined pressure. The constant oxygen pressures which were studied in turn were 0.18, 1.0, and 5.0% atm. The experimental set-up was essentially the same as that described under Section 3 above, except that the hearts were observed using polarized red light. Once again, ten intact diapausing pupae were used for the entire series of experiments.

TABLE IV

Effects of carbon monoxide at low oxygen tension on heartbeat of diapausing pupae

Ratio CO/O_2	5% atm. oxygen			1% atm. oxygen			0.18% oxygen		
	Average heartbeat index	Per cent inhibition	k(G)*	Average heartbeat index	Per cent inhibition	k(G)*	Average heartbeat index	Per cent inhibition	k(G)*
No CO	5.7			5.4			3.5		
3				5.4	0		1.6	53	2.4
10				3.5	35	19	0.8	76	3.2
18	5.6	2							
50				2.0	63	29	0.2	94	3.2
76	2.0	65	41						
99	1.3	78	28	0.9	83	20	0.1	97	3.0
555							0.0	100	
Average			34			23			2.9

* "Distribution constant" calculated from Warburg's partition equation (see text).

The first oxygen pressure to be studied in this manner was 0.18% atm. The pupae were equilibrated for eight hours with 0.18% atm. oxygen and 99.82% atm. of oxygen-free nitrogen. While holding the oxygen concentration constant, a certain percentage of carbon monoxide was then added to the flowing mixture of oxygen and nitrogen; namely, 0.54, 1.8, 9.0, 18.0, and 99.82% atm. carbon monoxide. The animals were equilibrated with each mixture for eight hours before recording the heartbeat. And before testing successive carbon monoxide mixtures, the system was ventilated for eight hours with 0.18% atm. oxygen and 99.82% atm. nitrogen.

The results are recorded in Table IV and the lower curve in Figure 3. It will be observed that in the absence of carbon monoxide the heartbeat index was already depressed by the low oxygen tension *per se*. As increasing percentages of carbon monoxide were added, one witnesses a further depression in heartbeat index attributable to carbon monoxide. Finally, at the low oxygen tension under consideration (0.18% atm.), the heartbeat is totally blocked at a CO/O_2 ratio of 555/1.

In like manner the experiment was repeated utilizing oxygen at the constant pressure of 1% atm. The results recorded in Table IV and Figure 3 show no depression attributable to the low oxygen pressure *per se*. However, as carbon monoxide is added, the heartbeat index begins to decline and continues to do so until one establishes a CO/O_2 ratio of 99/1—the highest ratio attainable at a total pressure of one atmosphere.

Finally, the experiment was repeated using oxygen at the constant pressure of 5% atm. A CO/O_2 ratio of 19/1 was the highest ratio attainable at one atmosphere total pressure. Therefore, the animals at this point were transferred to the transparent pressure chamber and the experiment continued at positive pressures up to five atmospheres. The results summarized in Table IV and Figure 3 show no inhibition attributable to the low oxygen pressure *per se*. Moreover, the carbon monoxide inhibition now becomes apparent only at high CO/O_2 ratios; *i.e.*, in excess of 18/1.

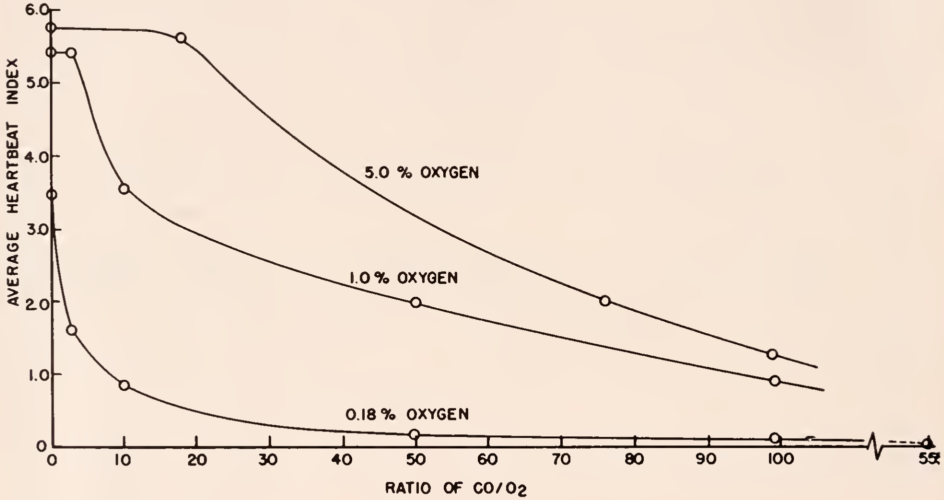


FIGURE 3. The effects of increasing concentrations of carbon monoxide on the heartbeat observed in intact diapausing Cecropia pupae. In the determination of each datum the experiment was continued for at least eight hours in order to compensate for the anaerobic reserve. The effects of carbon monoxide are dictated, not only by the CO/O_2 ratio, but also by the absolute tension of oxygen.

5. *Photoreversal of the CO-inhibition of the pupal heart*

By the same technique described in Section 4, nine diapausing pupae were equilibrated for eight hours with a flowing mixture of 1% atm. oxygen and 99% atm. carbon monoxide (CO/O_2 ratio of 99/1). The hearts were then examined in polarized red light and the heartbeat index calculated. All hearts were strongly inhibited, and four of the nine were not beating. Each heart, in turn, was then illuminated with a pair of focussed beams of intense white light, and the effects scored over a period of five minutes. The results recorded in Table V reveal a marked reversal of the carbon monoxide inhibition by white light.

As a control for the preceding experiment, the same group of animals was equilibrated for eight hours with a flowing mixture of 1% atm. oxygen and 99% atm. nitrogen. The heartbeat index was then recorded in red light and white light, as just described. No inhibition was observed either in red or in white light (Table V).

TABLE V

Light reversibility of the CO-inhibition of pupal hearts (within intact animal)

Animal No.	Rate (beats/min.) and amplitude* of heartbeat			
	99% CO 1% O_2 Red light	99% CO 1% O_2 White light	99% N_2 1% O_2 Red light	99% N_2 1% O_2 White light
1	0 (0)	6 (3)	3 (3)	4 (3)
2	3 (1)	7 (3)	11 (3)	16 (3)
3	0 (0)	14 (3)	4 (3)	7 (3)
4	1 (1)	3 (3)	3 (3)	3 (3)
5	0 (0)	9 (3)	3 (3)	13 (3)
6	3 (2)	3 (3)	4 (3)	4 (3)
7	0 (0)	4 (3)	8 (3)	6 (3)
8	2 (1)	9 (3)	3 (3)	2 (3)
9	1 (2)	2 (3)	5 (3)	4 (3)
Average heartbeat index**	0.44	5.7	4.6	5.9

* Amplitudes scored and recorded in parentheses after rate.

** See Harvey and Williams (1958) for calculation of average heartbeat index.

6. *Lethal effects of carbon monoxide at low oxygen pressure*

In the experimental results described above, normal heartbeat was maintained in nitrogen containing 1% atm. oxygen. By contrast, the heart was strongly inhibited in carbon monoxide containing 1% atm. oxygen. These results lead to the prediction that carbon monoxide should be a lethal agent for diapausing pupae when administered in combination with low oxygen pressure.

Five diapausing pupae were placed in each of two 3.5-liter Lucite pressure chambers along with a small dish of 10 per cent potassium hydroxide to absorb carbon dioxide. One chamber was flushed daily with ten volumes of a gas mixture containing 1% atm. oxygen in nitrogen; the other, with a mixture of 1% atm. oxygen in carbon monoxide. The experiment was continued for 14 days. The chambers were then opened and the animals examined. All of the control animals in nitrogen were lively and normal; all the experimental animals in carbon monoxide were flaccid and dead.

DISCUSSION

1. *The heartbeat of the adult moth*

The heartbeat of the adult moth is strongly inhibited by cyanide in that HCN, in a concentration of less than 10^{-5} *M*, inhibits the heartbeat by 50 per cent (Harvey and Williams, 1958). This finding is strong evidence that the heartbeat in the adult moth depends on the function of cytochrome oxidase.

In the present study the isolated adult heart was inhibited when the ratio of carbon monoxide to oxygen was 24/1 in the surrounding gas phase. This inhibition was promptly reversed by light. Moreover, the effectiveness of the light coincides with the absorption maxima of the cytochrome oxidase-CO complex. Therefore, the terminal oxidase of the adult heart may confidently be identified as cytochrome oxidase. This conclusion is consistent with the finding that the adult heart contains a high concentration of the several cytochromes including cytochrome oxidase (Shappirio and Williams, 1957a, 1957b).

2. *The heartbeat of the diapausing pupa*

As described in Sections 1 and 4 of the Results, it is easy to get the impression that the pupal heart differs from the adult heart in being insensitive to carbon monoxide. Even when the ratio of carbon monoxide to oxygen is raised as high as 19/1, the pupal heart continues to beat normally, provided that at least 5% atm. oxygen is present.

In the analogous case of the apparent insensitivity of the pupal heart to cyanide (Harvey and Williams, 1958), a satisfactory explanation was found to be the anaerobic capacity of the heart at this particular stage. However, this same explanation does not suffice to explain the insensitivity of the pupal heart to carbon monoxide. Thus, as we have seen (Table II), the pupal heart beats indefinitely in mixtures of carbon monoxide and oxygen (19/1), whereas it comes to a standstill after several hours in oxygen-free nitrogen. Therefore, on the basis of results of this type, one might be persuaded that the pupal heart fails to make use of CO-sensitive cytochrome oxidase, notwithstanding the presence in the pupal heart of a substantial titer of this same enzyme.

The present study makes clear that this conclusion is erroneous. It is our present purpose to show how the relatively high concentration of cytochrome oxidase in the pupal heart serves to camouflage the true sensitivity of the pupal heart to carbon monoxide.

3. *Pupal heartbeat at low oxygen tensions*

The resistance of the pupal heart to carbon monoxide can be accounted for most easily by first considering the insensitivity of the same heart to low oxygen tensions. The two phenomena, as we shall see, have a very close connection.

As illustrated in Figure 2, the heart of the diapausing pupa continues to beat normally for an indefinite period when the intact pupa is exposed to oxygen tensions as low as 0.5% atm. In experiments which are sufficiently prolonged to compensate for the anaerobic reserve of the pupal heart, one finds that the heartbeat is blocked in the total absence of oxygen and significantly depressed at external tensions lower than 0.5% atm. This implies that, after the exhaustion of its anaerobic reserve, the pupal heart is "micro-aerophilic." Oxygen is required, but a very low tension suffices. The fact that an external tension as low as 0.5% atm. sustains the normal heartbeat is especially impressive when one recalls that a tension of 0.5% atm. oxygen outside the insect corresponds to a still lower tension in the heart muscle itself.

In our opinion the micro-aerophilic character of the pupal heart can be accounted for in terms of the presence in the pupal heart of a substantial titer of cytochrome oxidase in conjunction with only a trace of its substrate *c*. Consider the situation in Figure 4A descriptive of the circumstances in the heart of the diapausing pupa. Here the trace of cytochrome *c* is diagrammed as passing electrons to the oxidized heme (+ + +) of cytochrome oxidase. The latter is present in the diapausing heart in great excess over cytochrome *c*. Provided that the trace of *c* has free access to the pool of oxidase, a steady-state will be established in which the reduction of oxidase is more than counterbalanced by the oxidation of oxidase by molecular oxygen. Therefore, the vast majority of cytochrome oxidase exists in the oxidized state. As diagrammed in Figure 5A, one can lower the oxygen tension, thereby establishing a new steady-state in which half of the oxidase is in the oxidized form and half in the reduced form. The respiration, as signalled by the rate of water formation, remains unimpaired.

The presence of the great excess of cytochrome oxidase has a further influence on the kinetics of this steady state. At very low oxygen tensions, most of the oxidase will be present in the reduced (+ +) state. This increases the concentration of reduced oxidase, the target of molecular oxygen, and permits a still lower oxygen tension to suffice. The excess oxidase acts, in effect, as a "buffer" against very low oxygen tensions. It is important to note that under all these circumstances the rate of electron transmission and of oxygen consumption (represented by the block marked H_2O in the diagrams) remain constant and independent of oxygen tension.

The set of circumstances, diagrammed in Figure 6A, considers the situation when the oxygen tension is so low as to limit the respiration. Almost all of the oxidase is now reduced. Finally, in a completely anaerobic situation, all the oxidase becomes reduced and the respiration is totally blocked at this point.

For these several reasons we can understand the reason why the pupal heart continues to beat normally in the presence of oxygen tensions as low as 0.5% atm. The explanation is found to lie in the disproportionately high concentration of cytochrome oxidase with respect to cytochrome *c*—the precise combination that characterizes the heart of the diapausing pupa (Shappirio and Williams, 1957a, 1957b). The surplus oxidase is "money in the bank" which can be drawn upon for transactions at low oxygen pressures.

4. *A theoretical consideration of CO-inhibition*

As Goddard (1947) points out, a mathematical treatment of the kinetics of the CO-inhibition of cytochrome oxidase requires so many simplifying assumptions that the formulation is scarcely descriptive of any real experiment. Indeed, we are unable to find in a search of the literature any satisfactory non-mathematical treatment. Consequently, we have been forced to develop a semi-diagrammatic presentation of the facts. Though descriptive of the experiments on the silkworm heart, we believe that the formulation will be pertinent to analogous studies.

A. *CO-inhibition at normal oxygen pressures*

The crux of the matter is that carbon monoxide combines only with the reduced form of cytochrome oxidase, the CO/O_2 ratio dictating the percentage of reduced oxidase which is complexed and inactivated. By virtue of the low concentration of

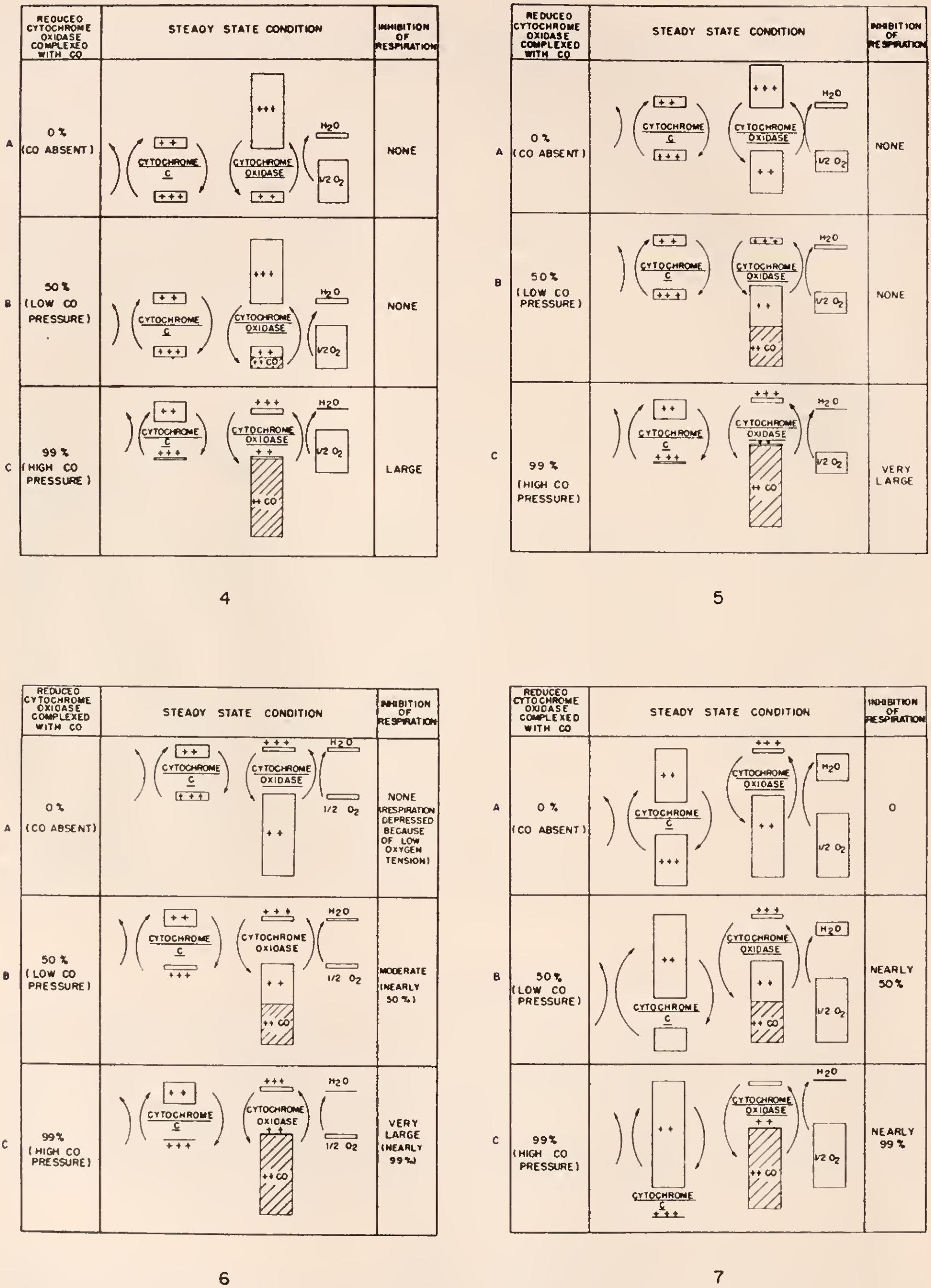

REDUCED CYTOCHROME OXIDASE COMPLEXED WITH CO
STEADY STATE CONDITION
INHIBITION OF RESPIRATION
A
0 % (CO ABSENT)
B
50 % (LOW CO PRESSURE)
C
99 % (HIGH CO PRESSURE)
CYTOCHROME C
CYTOCHROME OXIDASE
++
+++
++ CO
H_2O
1/2 O_2
NONE
LARGE
VERY LARGE
NONE (RESPIRATION DEPRESSED BECAUSE OF LOW OXYGEN TENSION)
MODERATE (NEARLY 50 %)
VERY LARGE (NEARLY 99 %)
0
NEARLY 50 %
NEARLY 99 %
4
5
6
7

FIGURE 4. CO-inhibition of the respiration of diapausing Cecropia pupae. Diagram of the steady-state condition of the electron transport system when the oxygen tension is not limiting respiration (5% atm. oxygen or above).

cytochrome *c* a correspondingly low concentration of reduced oxidase exists in the normal pupal heart at ordinary oxygen tensions (Fig. 4A). Under this circumstance carbon monoxide finds a limited target within the diapausing heart. The addition of sufficient carbon monoxide to complex, say, 50 per cent of the reduced oxidase transiently slows the rate of oxidation of reduced cytochrome oxidase. This causes additional oxidase to accumulate in the reduced form until the amount of reduced oxidase is twice that present in the absence of carbon monoxide (Fig. 4B). Although 50 per cent continues to be complexed by carbon monoxide, in the new steady-state the amount of uncomplexed reduced oxidase becomes the same as it had been in the total absence of carbon monoxide. Consequently, the respiration is uninhibited.

At very high pressures of carbon monoxide sufficient to complex, say, 99 per cent of reduced oxidase, the reserve of oxidase becomes limiting and the system can no longer undergo the necessary degree of internal compensation (Fig. 4C). Therefore, the rates of electron transfer, oxygen consumption, and water formation are slowed down.

B. *CO-inhibition at low oxygen pressures*

As we have just seen, the concentration of reduced cytochrome oxidase and the sensitivity to carbon monoxide can be enhanced experimentally by lowering the oxygen tension. Let us consider the hypothetical case where the oxygen pressure is lowered until 50 per cent of the oxidase is in the reduced form (Fig. 5A). The rate of oxygen consumption and water formation remains the same as at higher oxygen pressures.

If one now adds enough carbon monoxide to complex 50 per cent of the reduced oxidase, a new steady-state results in which nearly all the oxidase shifts to the reduced condition (Fig. 5B). Whether the respiration will be inhibited will be determined by whether sufficient oxidase is present to supply the necessary degree of compensation. In the case considered in Figure 5B, this condition is fulfilled and the rate of water formation is diagrammed as uninhibited. However, as shown in Figure 5C, the compensatory mechanism breaks down if the pressure of carbon monoxide is further increased. A strong inhibition of respiration and of water formation is then observed.

C. *CO-inhibition at very low oxygen pressures*

Attention is finally directed to the set of circumstances diagrammed in Figure 6. The oxygen pressure at the outset is reduced to a very low level (0.18% atm.) so that the respiration is already inhibited and most of the cytochrome oxidase is present in the reduced form (Fig. 6A). The reserves of oxidized

FIGURE 5. CO-inhibition of the respiration of diapausing Cecropia pupae. Diagram of the electron transport system when the oxygen tension is low but not limiting respiration (1% atm. oxygen).

FIGURE 6. CO-inhibition of the respiration of diapausing Cecropia pupae. Diagram of the electron transport system when the oxygen tension is limiting respiration (0.18% atm. oxygen).

FIGURE 7. CO-inhibition of the respiration of developing adults of the Cecropia silkworm. Diagram of the steady-state condition of the electron transport system when the oxygen tension is not limiting the respiration. The resynthesis of cytochrome *c* enhances the metabolism and the sensitivity to carbon monoxide.

oxidase have already been exhausted and the system is immediately sensitive to low pressures of carbon monoxide (Figs. 6B and 6C).

5. *Carbon monoxide and the pupal heart*

Under experimental conditions the pupal heart is found to respond to carbon monoxide in accordance with theory. In Figure 8 the experimental results of

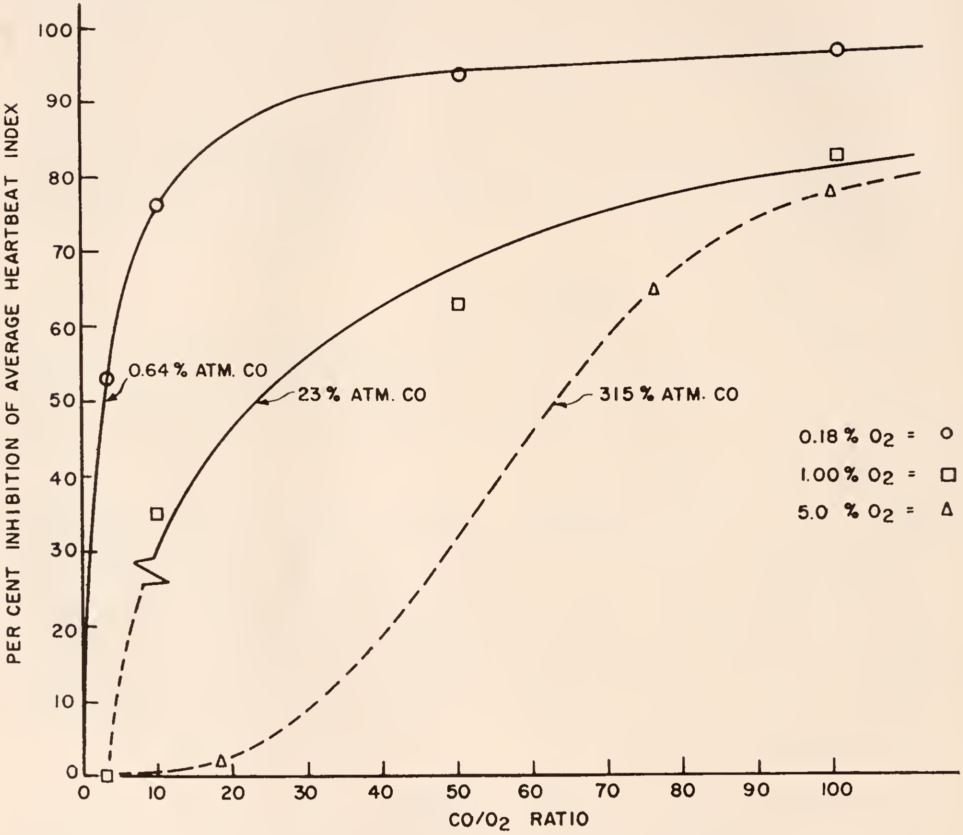


FIGURE 8. Per cent inhibition of the heartbeat of diapausing Cecropia pupae as a function of the CO/O_2 ratio. The inhibition is determined, not only by the CO/O_2 ratio, but also by the absolute tension of oxygen. The uppermost curve has been drawn in accordance with the Warburg equation.

Table IV and Figure 3 have been brought together and plotted on a scale in which the per cent inhibition of heartbeat is considered as a function of the CO/O_2 ratio at each of three oxygen pressures.

At the very low oxygen pressure of 0.18% atm., the heart is inhibited by any finite pressure of carbon monoxide and 50 per cent inhibited by carbon monoxide at a pressure of only 0.64% atm. (5 mm. Hg). When the oxygen pressure is raised to 1% atm., then the carbon monoxide pressure must be increased to 23%

atm. to achieve 50 per cent inhibition. Finally, at the still higher oxygen pressure of 5% atm., 50 per cent inhibition is brought about only by a very high pressure of carbon monoxide (315% atm.).

6. *The critical effects of cytochrome c*

The analysis, up to this point, has focussed attention on the oxidation of cytochrome oxidase by molecular oxygen. In a given system at low oxygen pressures, the pressure of oxygen dictates the partition of oxidase between oxidized and reduced states; this partition, in turn, is found to condition the sensitivity to carbon monoxide. An examination of Figure 7 will make clear that this same partition can be influenced at any specific oxygen pressure by varying the rate of reduction of cytochrome oxidase. This is achieved by increasing the concentration of reduced cytochrome *c*.

It is precisely this circumstance which supervenes at the initiation of adult development. As Shappirio and Williams (1957a, 1957b) have shown, a rapid synthesis of cytochrome *c* occurs at this time. The metabolism is enhanced and now shows a definite sensitivity to carbon monoxide even at high oxygen pressures (Schneiderman and Williams, 1954a). This result is intelligible in terms of the discussion set forth above.

Shappirio and Williams (1957b) have been able to duplicate this same phenomenon *in vitro* by the addition of extrinsic cytochrome *c* to washed homogenates of diapausing pupal tissues (using DPNH as substrate). The metabolism of the homogenate increases markedly and shows a substantial sensitivity to inhibitors of cytochrome oxidase.

7. *Warburg's equation for CO-inhibition*

In the hypothetical situation where all the cytochrome oxidase is in the reduced condition, then, as Warburg (1927) points out, one can formulate a simple stoichiometric relation between the inhibition of respiration and the CO/O_2 ratio.

$$\frac{\text{Respiration uninhibited by CO}}{\text{Respiration inhibited by CO}} = K\frac{O_2}{CO}$$

In practice it is difficult or impossible to establish a steady-state in which all the oxidase is reduced. Biochemists have routinely attempted to satisfy this requirement by the addition to *in vitro* systems of a large excess of substrates and cytochrome *c*.

The present investigation directs attention to a far simpler solution of the problem which is applicable, not only to *in vitro* systems, but also to intact organisms. This technique, as we have seen, is to favor the reduction of oxidase by working at very low oxygen pressures. Under this circumstance the quantitative aspects of the carbon monoxide inhibition are in precise agreement with Warburg's formulation. This fact is evident in Figure 8 where the uppermost curve is a theoretical curve constructed according to the Warburg equation, the constant K being 3. This low value implies that carbon monoxide has a higher affinity for reduced cytochrome oxidase than is customarily assumed.

8. *The terminal oxidase during metamorphosis*

We are persuaded by the argument outlined above that the heartbeat of the diapausing pupa is sustained by the CO-sensitive cytochrome oxidase. Confirmation of this view is found in the demonstration that the CO-inhibition of the pupal heartbeat is promptly reversed by light (Table V). The reason that the pupal heart appears to be insensitive to carbon monoxide is that the true sensitivity is camouflaged by the great excess of cytochrome oxidase that is present. In our opinion many additional instances of so-called CO-insensitive respiration reported in the literature will be found to have a similar basis.

In the earlier studies we have interpreted the CO-resistance of the diapausing pupa to signal the presence of a CO-insensitive oxidase. However, it is worth recalling that the non-muscular tissues of the diapausing pupa also contain cytochrome oxidase in great excess over cytochrome *c* (Shappirio and Williams, 1957a and b). Therefore, if cytochrome oxidase can give the false impression of CO-insensitivity in the heart, there is no *a priori* reason why it cannot do so in the non-muscular tissues. In this connection it is of interest that the pupa as a whole is killed by exposure to carbon monoxide at low oxygen pressures; *i.e.,* under conditions where the excess of cytochrome oxidase is eliminated from the field of action (Section 6 of Results).

The present study of the heart does not permit a clear decision as to the role of cytochrome oxidase in the pupa as a whole. Most fortunately, however, this particular matter has simultaneously been studied in an independent investigation by Kurland and Schneiderman, and will be considered in detail in a forthcoming publication.

SUMMARY

1. The heartbeat of the adult Cecropia moth is inhibited by suitable pressures of carbon monoxide, and this inhibition is reversed by light.

2. The wave-lengths which are maximally effective in reversing the CO-inhibition are in good agreement with the absorption maxima of the CO-cytochrome oxidase complex.

3. Therefore, the terminal oxidase of the adult heart may be identified as cytochrome oxidase.

4. The situation is much more complex in the case of the diapausing pupa. The latter shows a considerable capacity for anaerobic metabolism, and its heart can beat for several hours in the total absence of oxygen. Moreover, normal heartbeat continues indefinitely in the presence of oxygen pressures as low as 0.5% atm.

5. After exhaustion of the "anaerobic reserve," the pupal heart may be said to be "micro-aerophilic." Oxygen is required but a remarkably low tension suffices.

6. At ordinary oxygen pressures such as that in air, it is difficult or impossible to demonstrate any sensitivity of the pupal heart to carbon monoxide. However, when the oxygen tension is decreased to very low levels, the heartbeat shows a clear sensitivity to carbon monoxide. Thus, at the low oxygen tension of 0.18% atm., the pupal heartbeat is inhibited 50 per cent by carbon monoxide at a pressure of only 5 mm. Hg.

7. Under these circumstances, the inhibition of the pupal heartbeat is light-reversible.

8. The actions of oxygen and carbon monoxide are considered in detail. The terminal oxidase of the pupal heart is found to be cytochrome oxidase.

9. The resistance of the pupal heart to carbon monoxide and to low oxygen pressures can be accounted for in terms of the presence in the pupal heart of a great excess of cytochrome oxidase relative to cytochrome *c*.

LITERATURE CITED

Ephrussi, B., and G. W. Beadle, 1936. A technique of transplantation for *Drosophila*. *Amer. Nat.,* **52**: 218–225.

Goddard, D. R., 1947. Respiratory enzymes. *In:* Physical Chemistry of Cells and Tissues, (R. Höber, editor), London. J. and A. Churchill, Ltd.: 397–420.

Harvey, W. R., and C. M. Williams, 1958. Physiology of insect diapause. XI. Cyanide-sensitivity of the heartbeat of the Cecropia silkworm, with special reference to the anaerobic capacity of the heart. *Biol. Bull.,* **114**: 23–35.

Hill, R., and E. F. Hartree, 1953. Hematin compounds in plants. *Ann. Rev. Plant Physiol.,* **4**: 115–150.

Melnick, J. L., 1942. The photochemical spectrum of cytochrome oxidase. *J. Biol. Chem.,* **146**: 385–390.

Schneiderman, H. A., and C. M. Williams, 1954a. The physiology of insect diapause. VIII. Qualitative changes in the metabolism of the Cecropia silkworm during diapause and development. *Biol. Bull.,* **106**: 210–229.

Schneiderman, H. A., and C. M. Williams, 1954b. The physiology of insect diapause. IX. The cytochrome oxidase system in relation to the diapause and development of the Cecropia silkworm. *Biol. Bull.,* **106**: 238–252.

Schneiderman, H. A., and N. Feder, 1954. A respirometer for metabolic studies at high gaseous pressures. *Biol. Bull.,* **106**: 230–237.

Shappirio, D. G., and C. M. Williams, 1957a. The cytochrome system of the Cecropia silkworm. I. Spectroscopic studies of individual tissues. *Proc. Roy. Soc. London, Ser. B,* **147**: 218–232.

Shappirio, D. G., and C. M. Williams, 1957b. The cytochrome system of the Cecropia silkworm. II. Spectrophotometric studies of oxidative enzyme systems in the wing epithelium. *Proc. Roy. Soc. London, Ser. B,* **147**: 233–246.

Warburg, O., 1927. Über die Wirkung von Kohlenoxyd und Stickoxyd auf Atmung und Gärung. *Biochem. Zeitschr.,* **189**: 354–380.

Warburg, O., 1949. Heavy metal prosthetic groups and enzyme action. Oxford at the Clarendon Press. pp. 230.

Williams, C. M., 1946. Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia. Biol. Bull.* **90**: 234–243.

Winzler, R. J., 1941. The respiration of bakers' yeast at low oxygen tension. *J. Cell. Comp. Physiol.,* **17**: 263–276.

NOTES ON THE BIOLOGY OF THE FIVE-LUNULED SAND DOLLAR

LIBBIE H. HYMAN

American Museum of Natural History, New York 24, N.Y.

The five-lunuled sand dollar, *Mellita quinquiesperforata* (Leske, 1778), is abundant on the sand flats at Beaufort, North Carolina, where the following observations were made during a short stay in August, 1957, at the Duke University Marine Laboratory.

Burrowing. The animal lives in shallow water in a horizontal position, usually completely covered by a thin layer of sand although sometimes the posterior lunule remains visible. Specimens observed were motionless although the animal can progress under the sand. On being removed and placed on top of the sand under water it promptly burrows under again. The statements about its method of burrowing in Pearse, Humm and Wharton (1942) are totally at variance with my observations and appear wholly erroneous. They say that in burrowing the animal moves directly downward, waving its spines and tube feet so as to move sand from beneath the test to the upper surface, and does not progress anteriorly as it descends. My observations on animals in their natural habitat agree with those of Kenk (1944) on the closely related *Mellita lata* at Puerto Rico. Unlike regular urchins, sand dollars and other irregular urchins are polarized with definite anterior and posterior ends and can move only in the anterior direction, that opposite the posterior lunule in the present species. When placed on the sand under water in its natural habitat *Mellita* promptly begins to bury itself. It does this by simply advancing into the sand in a horizontal or slightly oblique orientation, using its ordinary method of locomotion by waves of movement along the spines of the oral surface. Kenk gives a succession of photographs showing *Mellita* advancing into the sand, which rapidly covers it. I did not time the reaction but only a couple of minutes is required for the five-lunuled species to cover itself with a thin layer of sand whereupon it comes to a halt. It positively does not move downward, does not remove sand from beneath it, and does progress forward. It is improbable that the tube feet play any role in locomotion as they are small and feeble and of limited distribution.

Lunules. Contrary to the statement of the Berrills (1957), in *Mellita* sand positively does not pass through the lunules from the oral to the aboral surface during the burrowing process. To the contrary as the animal advances into the sand, sand falls through the lunules to the oral surface, thus if anything retarding the burying process. It is known for a Japanese species (Ikeda, 1941) that the lunules are important in burrowing and righting but this is definitely not the case in *Mellita.* The further suggestion of the Berrills that the lunules strengthen the test by fusing the oral and aboral surface is extremely improbable because the two surfaces of the test are so much connected by interior calcareous columns that little space remains for the viscera of the animal. The function of the lunules in the five-lunuled sand dollar remains enigmatical. When the animal is placed in a bowl of sea water without sand the spines along the lunules are seen in constant activity but no current could be detected passing through the lunules. If the animal is lifted out of water these spines bend so as to close the lunules. If the animal is placed in a clean bowl of sea water in order to view the oral surface and the aboral surface covered with sand, to make the animal more comfortable, so to speak, sand falls through the lunules as the animal peregrinates around the bowl and covers the bottom, preventing a view of the oral surface. It is difficult to ascribe any value in nature to this behavior.

Podia. Very little information is available in the literature concerning the distribution of the podia or tube feet of sand dollars, apart from the specialized feet of the aboral petaloids. About the only detailed reports are those of Gregory (1911) and Parker and Van Alstyne (1932) for the New England sand dollar, *Echinarachnius parma;* here the podia are said to occur abundantly along the radii of both oral and aboral surfaces and also around the entire margin. Unlike the situation in regular urchins where each podium passes through two holes in the test, in sand dollars there is one hole for each podium. It does not seem to be generally realized that the distribution of the podia in any sand dollar can be determined by examining the cleaned test under low magnification whereupon the small holes through which the podia emerge are seen. Kenk (1944) stated that in *Mellita lata* podia occur along the margins of the lunules and among the spines of the entire circumference as well as along the ambulacral furrows of the oral surface. In the five-lunuled *Mellita* repeated and careful examinations of cleaned tests revealed the small holes for the podia *only* along the ambulacral furrows of the oral surface. They are definitely absent from the margins of the lunules and the edge of the test. These furrows lack spine tubercles that are present everywhere else on the test. Despite repeated efforts I was never able to see the podia on living animals turned oral side up in a dish of sea water under magnification. Presumably under such abnormal conditions the podia are retracted. The pedicellariae are easily seen, constantly jerking about, and may have been mistaken for podia by some observers. They are very numerous on the areas between the ambulacral furrows and this is rather curious because Mortensen (1948) declares that he could rarely find pedicellariae in *Mellita.*

Feeding. My main purpose was to observe the feeding process but in this I failed signally. The only observations on the feeding of sand dollars are those of the MacGinities (1949). They state that the cilia of the aboral spine bases create currents that carry food particles entrapped in mucus to the posterior edge where they are passed to the oral side and proceed along the ambulacral furrows to the mouth. In reply to inquiries, Dr. MacGinitie kindly stated that the species used was *Dendraster excentricus* (or a variant thereof), that juveniles are best for observations of this kind, and that they must be acclimated to laboratory conditions for a week or more. Inspection of the cleaned tests of *Dendraster excentricus* showed that the podial pores are limited to the ambulacral furrows and that these furrows are devoid of spines. MacGinitie does not seem to have observed the podia and when questioned as to the mechanism of movement of the mucous-coated food particles along the ambulacral furrows replied that it is ciliary. I am of the opinion that the podia must be the chief mechanism of food movement along the furrows as they seem to have no other function, not being concerned in locomotion.

My observations were made on freshly collected specimens placed in shallow dishes of sea water without sand. Carmine grains and bits of crushed plankton (kindly supplied by Dr. Frank Maturo), untreated or stained with neutral red or mixed with carmine grains, were placed on various sites on the aboral or oral surfaces, including the ambulacral furrows, and observed over a long period. No trace of movement of the particles was seen in any location nor was there any evidence of ciliary currents anywhere. This negative result is probably to be attributed to the abnormal conditions under which the animals were kept. Unfortunately it did not occur to me to examine the spines microscopically for the presence of cilia. A morsel of plankton placed among the marginal spines caused commotion among them; they converged on the morsel and manipulated it for a long time but it was never moved to the slightest extent from the site on which it had been placed. Evidently to determine the mode of feeding of *Mellita* will require time, patience and ingenuity. It is difficult to think of any method by which these animals can be kept in normal conditions that at the same time will permit a view of the oral surface.

The digestive tract of one specimen was removed and the contents examined under the compound microscope. The intestine contained no sand grains nor any coarse material nor any remains of larger organisms. The contents consisted entirely of multitudes of minute naked organisms, all seemingly of one kind, apparently nannoplankton.

LITERATURE CITED

Berrill, N. J., and J. Berrill., 1957. 1001 Questions Answered About the Seashore. Dodd, Mead, and Company, New York; xii + 305 pp.

Gregory, Emily, 1911. Observations on the water-vascular system of *Echinarachnius parma*. *Zool. Anz.,* **38**: 323–326.

Ikeda, M., 1941. Function of the lunules of *Astriclypeus* as observed in the righting movement (Echinoidea). *Annot. Zool. Japon.,* **20**: 78–82.

Kenk, R., 1944. Ecological observations on two Puerto Rico echinoderms, *Mellita lata* and *Astropecten marginatus*. *Biol. Bull.,* **87**: 177–187.

MacGinitie, G. E., and N. MacGinitie., 1949. Natural History of Marine Animals. McGraw-Hill Book Company, New York; xii + 473 pp.

Mortensen, Th., 1948. A monograph of the Echinoidea, vol. IV, part 2. C. A. Reitzel, Copenhagen; 471 pp.

Parker, G. H., and M. Van Alstyne, 1932. Locomotor organs of *Echinarachnius parma*. *Biol. Bull.,* **62**: 195–200.

Pearse, A. S., H. J. Humm and G. W. Wharton, 1942. Ecology of sand beaches at Beaufort, N. C. *Ecol. Monogr.,* **12**: 137–190.

SOME ASPECTS OF BEHAVIOR OF OYSTERS AT DIFFERENT TEMPERATURES

V. L. LOOSANOFF

U. S. Fish and Wildlife Service, Milford, Conn.

The oyster, *Crassostrea virginica* Gmelin, is a sedentary mollusk which must pump definite quantities of water through its gills, to obtain the food and oxygen necessary for its existence, and to get rid of the waste products. The stream of water created by a pumping oyster can easily be seen if the latter is kept in a shallow tray in water just deep enough to cover it. Even casual observations will show that the quantity of water pumped by an oyster changes from time to time, and that different oysters may pump at different rates.

In keeping oysters under laboratory conditions it is, obviously, of advantage to know the approximate quantity of water needed for their normal existence. The same information is of practical use to oyster cultivators in deciding the number of oysters that can be planted in a certain area to achieve the best growth. Furthermore, because of the recent progress in the artificial propagation of bivalves (Loosanoff and Davis, 1950; Loosanoff, 1954), which is now leading to mass production of clams and oysters under hatchery conditions, and because of the recent interest in the utilization for shellfish culture of small, salt water ponds, where annual fluctuations in temperatures may be great, extending in some regions from nearly 0.0° to 32.0° C. or even higher, a more complete knowledge of the behavior of the mollusks within this temperature range is needed. Finally, the differences in the rates of water pumping at the same temperatures by oysters from different geographic areas may be used as the criterion to ascertain the existence of different physiological races.

Studies of various aspects of filtration of the filter-feeding invertebrates, including oysters and closely related mollusks, have been made by many investigators. Comprehensive reviews of several hundred articles on these subjects were recently offered by Verwey (1952) and Jørgensen (1955); therefore, only those dealing directly with rates of water pumping by bivalves, especially oysters, will be referred to here.

The first comprehensive studies of the rate of pumping of the American oyster, *C. virginica,* at different temperatures were made by Galtsoff (1928a, 1928b). He used two methods. The first, the so-called "tank method," was designed primarily to collect the water after it had passed through the gills. The second or "carmine method" was devised to measure the rate of movement of the column of water flowing from the exhalant chamber through a glass tube. Galtsoff concluded that the maximum flow of water produced by an adult oyster, three to four inches in length, was 3.9 liters per hour at a temperature of 25.0° C. Galtsoff's conclusions were challenged by Nelson (1935, 1938) who considered these figures too low and thought that the methods used by Galtsoff unfavorably affected the experimental animals. Nelson, therefore, believed that Galtsoff's data did not truly present the normal activities of oysters. Nelson (1935) also reported that, instead of the 3.9 liters per hour indicated by Galtsoff as the maximum quantity of water that can be pumped by an oyster, some of the animals in his experiments pumped water at the rate of approximately 26.0 liters per hour. In our own work, we have recorded pumping rates of individual oysters as high as 34.0 liters per hour, *i.e.,* about ten times greater than Galtsoff's maximum (Loosanoff and Nomejko, 1946).

Most of Nelson's observations, as well as ours and those of several other investigators, were made, however, while studying various aspects of the physiological behavior of oysters and not during the studies devoted primarily to the evaluation of the effects of temperature upon their pumping rate. Since the need of more complete information on this subject still existed, the experiments discussed in this paper were conducted.

I wish to express my appreciation to Charles A. Nomejko for tabulating some of the data and for preparing photographs of the kymograph records, to Barbara J. Myers for the statistical treatment of the data used in this article and to Rita S. Riccio for her help in preparing the manuscript.

Methods

Only Long Island Sound oysters were used in these studies. For the sake of uniformity, they were selected to approximate the following standards: length—between 100.0 and 110.0 mm.; width—between 80.0 and 85.0 mm.; depth—between 30.0 and 35.0 mm.; and volume—between 85.0 and 100.0 cc. Before the oysters were used they were conditioned for several days at the temperature to be employed in the experiment.

The method for measuring the rate of flow of water through the gills of the oysters was based on the suggestions and apparatus of Moore (1910), Galtsoff (1926) and Nelson (1936), and was fully described in our article on feeding of oysters in relation to different concentrations of micro-organisms (Loosanoff and Engle, 1947). Here it is sufficient to mention that the so-called "rubber apron" method interfered in no way with the normal activities of the oysters and allowed us to collect and measure accurately the quantities of water pumped by them. Both the rate of pumping and the shell movements of the experimental oysters were continuously recorded by kymographs.

The temperature range covered by these experiments extended from about 0.0° to 38.0° C. The temperatures were usually maintained within ± 0.5° C., and the temperature intervals were 2.0° C. apart. The salinity of the water was usually about 27.0 p.p.t. and the pH, about 7.7.

In all these experiments the oysters were kept in running water, a condition which assured a more normal behavior than if the oysters had been confined to a small container repumping, time after time, the same water which, eventually, could become heavily laden with excretory products. The periods of observation extended from five to seven hours. Therefore, the conclusions offered here refer to these comparatively short periods, and the rates of pumping, as found in these experiments, would not necessarily be representative of longer exposures.

RESULTS

Our studies, an abstract of which has already been offered (Loosanoff, 1950), consisted of 478 individual observations. However, because many oysters either did not open at all or opened but did not pump water, the conclusions are based, actually, on 337 experimental records (Table I).

The lack of activity, as could be expected, was virtually confined to the lower temperatures. Below 2.0° C. only one of eight oysters pumped. It opened its shells when the temperature was only 1.2° C. and, regardless of the somewhat irregular and feeble shell movements, it produced a weak but, nevertheless, real flow of water. The ability to pump water at low temperature was also recorded for another individual that pumped a relatively large quantity of water at a temperature between 2.7° and 3.3° C. and maintained such a pumping rate for several hours (Fig. 1). Hopkins (1933) working on the closely related species, *Crassostrea gigas,* also reports that the distinct flow produced by this mollusk was observed and recorded at as low a temperature as 2.6° C.

TABLE I

Average rates of water pumping, in cc. per hour, of groups of oysters subjected for approximately six-hour periods to temperatures ranging from 0.0° to 38.0° C.

Temperature intervals ° C.	Number of oysters			Pumping rate	
	Total	Open	Pumping	Average	Maximum
0.0– 2.0	8	2	1	113	—
2.1– 4.0	52	22	1	863	1,020
4.1– 6.0	28	14	5	180	266
6.1– 8.0	20	12	6	495	1,197
8.1–10.0	36	21	16	763	1,594
10.1–12.0	36	24	22	2,914	4,303
12.1–14.0	32	25	22	3,902	5,409
14.1–16.0	28	27	26	4,344	5,787
16.1–18.0	21	21	21	9,083	11,583
18.1–20.0	33	33	33	7,020	9,773
20.1–22.0	42	42	42	9,802	13,341
22.1–24.0	24	24	24	7,795	10,802
24.1–26.0	14	14	14	9,537	12,635
26.1–28.0	20	20	20	9,366	11,569
28.1–30.0	24	24	24	12,983	15,155
30.1–32.0	19	19	19	11,813	16,253
32.1–34.0	20	20	20	8,948	12,856
34.1–36.0	17	17	17	2,785	4,201
36.1–38.0	4	4	4	2,449	3,884
Total	478	385	337	—	—

Since some of the oysters exposed to very low temperatures not only kept their shells open, but also pumped small quantities of water, experiments were devised to ascertain whether these oysters were ingesting food under such conditions. They were conducted during February when the temperature of Milford Harbor water is at its lowest. Three-year-old oysters, brought from outdoors, were placed into individual glass dishes which, in turn, were placed in shallow, white enamel trays of cold, running sea water. A cold culture of deep-green *Chlorella* was added as a biological indicator, to appear in the feces if the oysters fed.

Of the 90 oysters kept for 24 hours at a temperature ranging between 2.0° and 3.0° C., only one individual passed a small quantity of true feces. However, about 15 per cent of the oysters formed pseudo feces. When the temperature was kept between 3.0° and 4.0° C., approximately one-half of the 90 oysters formed pseudo feces, but only a single case of true feces was observed. Thirty oysters from this group were opened and examination showed that in all but one case their stomachs were empty and crystalline styles absent. The same results were obtained when the temperature ranged between 4.0° and 5.0° C. However, when the temperature was kept between 5.0° and 6.0° C., 11 of the 90 oysters expelled true feces, two of them in large quantities, and over 75 per cent formed pseudo feces.

These experiments have shown that, although feeding of oysters below 5.0° C. occurs only as an exception, a relatively high percentage may form pseudo feces, even at a temperature as low as 2.0° or 3.0° C. The explanation as to why pseudo feces can be produced at lower temperatures than true feces lies, perhaps, in the observation made by Galtsoff (1928a), that the frontal cilia are able to transport the particles along the surface of the gills at a temperature of only about 3.0° C., while the lateral cilia can, as a rule, produce a current only when the temperature of the surrounding water is about 5.0° C.

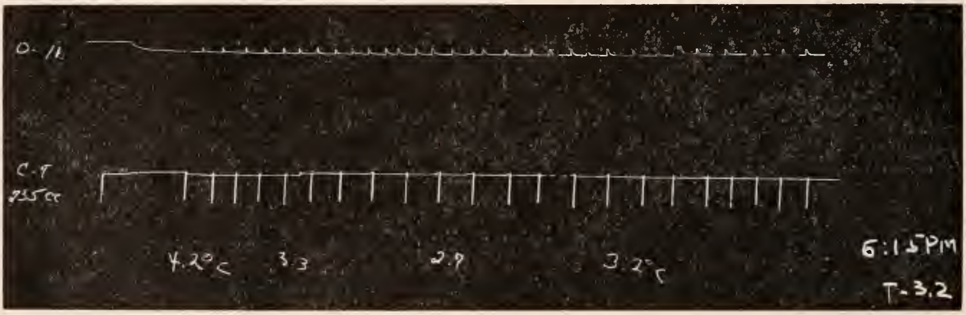


FIGURE 1. Kymograph record of shell movements (1st line) and rate of pumping (2nd line) of an oyster exposed to water temperature ranging between 2.7° and 4.2° C. from 11:45 A.M. to 6:15 P.M. Each vertical mark of the second line designates the discharge of 235 cc. of water pumped by the oyster.

The observations that some oysters feed at low temperatures are supported by those made by Nomejko and Chanley of Milford Laboratory who, in conducting a survey in Long Island Sound on March 11, 1954, dredged an oyster which possessed a well-developed crystalline style and contained food in its stomach. The temperature of the water at that time was approximately 3.0° C.

Our studies also showed that many oysters which open at the lower temperature may move their shells steadily, but pump no water. Twenty-one of the 22 oysters which opened at temperatures between 2.1° C. and 4.0° C. behaved in this manner (Table I).

Our observations lead us to believe that Galtsoff's (1928a) statement, that in

C. virginica no current is produced and no feeding occurs at or below 5.0° C., should be qualified because this rule, obviously, does not apply to all individuals. His generalizations, nevertheless, remain applicable to the large majority of the oysters.

The average and maximum rates of pumping of the groups of oysters within each temperature interval are given in Table I. In general, the rate of pumping remained low until the temperature interval of 8.0°–10.0° C. was reached. After that, and until the temperature of about 16.0° C. was attained, it showed a moderate increase. No radical fluctuations were recorded, however, between this point and the temperature of 28.0° C. The greatest average rate of pumping, 12,983 cc. per hour, was recorded between 30.0° and 32.0° C. After passing the maximum, the relatively fast pumping continued until about 34.0° C. and then abruptly decreased.

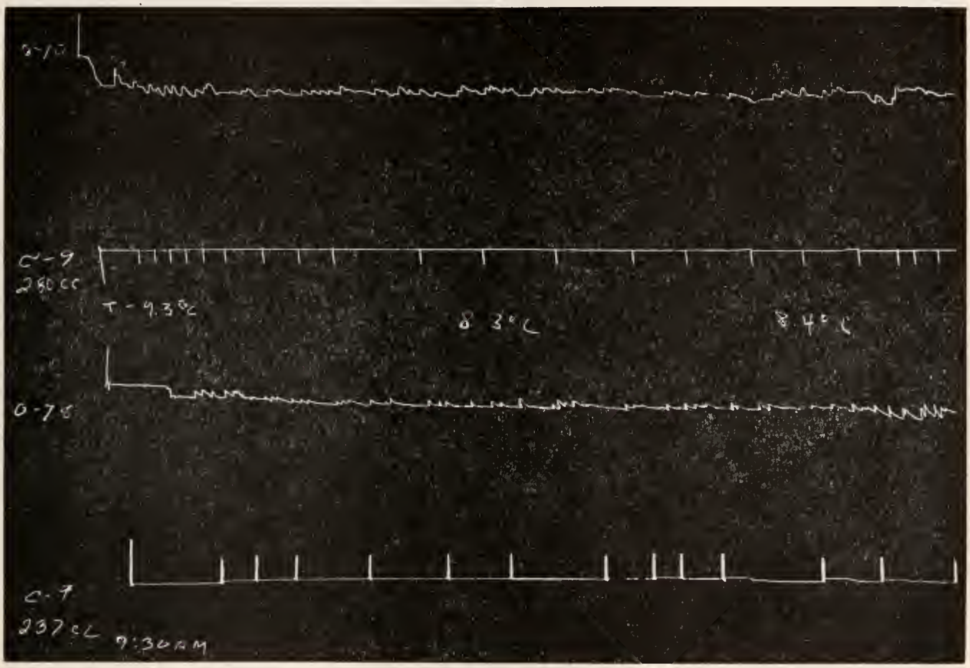


FIGURE 2. Kymograph record showing shell movements (1st and 3rd lines) and rates of pumping (2nd and 4th lines) of two oysters exposed to temperatures ranging from 8.3° to 9.3° C. from 9:30 A.M. to 2:15 P.M. Each vertical mark on the second line designates the discharge of 280 cc. of water pumped by the oysters, while each mark of the fourth line shows the discharge of 237 cc.

Some aspects of pumping at different temperatures are demonstrated in this article by photographs of the kymograph records showing both shell movements and rates of pumping. At comparatively low temperatures, such as 8.0° to 9.5° C., shell movements of the oysters may not be well defined and the amount of water pumped through the gills remains relatively small (Fig. 2). As the temperature of the water increases toward the optimum range, many oysters begin to display the type of shell movement called "staircase" or "treppe," which some students think is due to chemical stimulation affecting the experimental oysters with a gradual increase in intensity. We hesitate to accept this as a satisfactory explanation of the phenomenon. In many of our experiments the "staircase" shell movement occurred in normal-feeding oysters, although, as shown in Figure 3, this type of shell movement was often observed soon after the oysters opened and began to pump.

In our studies usually two or four oysters were under simultaneous observation, receiving the same amount of water, connected to the same kymograph and treated identically in all other respects. Yet, in many instances, including the one shown in Figure 4, the "staircase" type of shell movement was displayed at any given time by one oyster only. This clearly indicated that it was not a specific chemical in the water that was responsible for stimulation of the oysters leading to this type of shell movement. Obviously, further physiological studies are needed to explain the cause of the "staircase" type of shell movement.

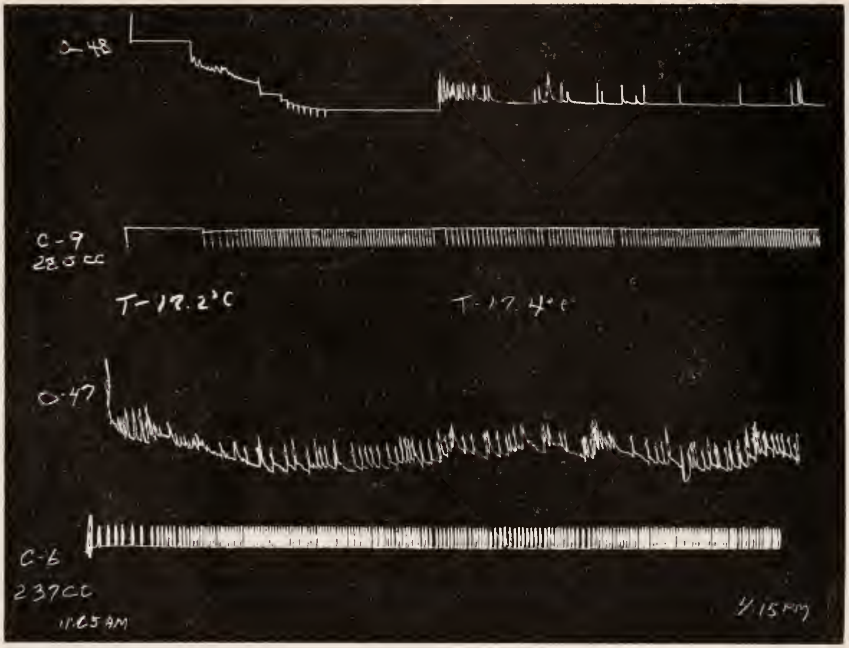


Figure 3. Kymograph record showing shell movements (1st and 3rd lines) and rates of pumping (2nd and 4th lines) of two oysters exposed to temperatures ranging from 17.2° to 17.4° C. from 11:05 A.M. to 4:15 P.M. Each vertical mark on the second line designates the discharge of 280 cc. of water pumped by the oysters, while each mark of the fourth line shows the discharge of 237 cc.

Within the favorable temperature range many oysters were recorded as pumping over 20,000 cc. per hour, and several individuals were observed pumping at the rate of 25,000 to 29,000 cc. per hour. The maximum rate of pumping for an individual oyster was recorded at a temperature ranging between 24.1° and 24.5° C. when one of the two oysters (indicated as O-10 in Figure 4) averaged 37,446 cc. per hour for a period of about five hours, and for several shorter periods of about 15 minutes pumped at the rate of about 40,000 cc. per hour. Because of such a rapid rate of pumping the strokes of the needle on the kymograph drum, each representing a discharge of 237 cc. of water, were made so close to each other that the record appeared blurred. Fortunately, by employing the low power of a dissecting microscope the strokes could be accurately counted and the record properly analyzed and evaluated.

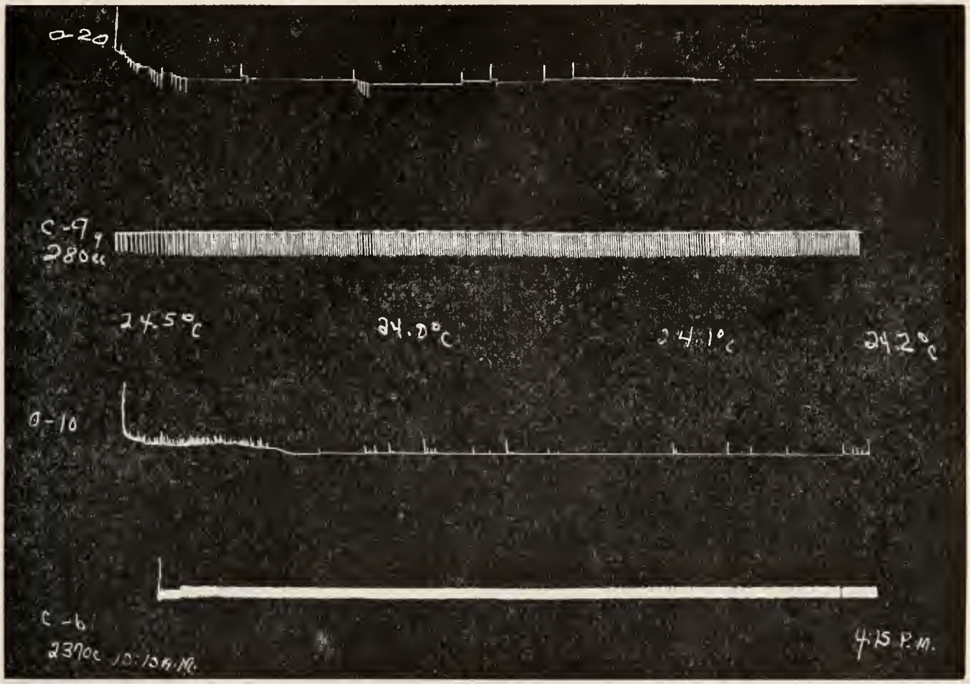


FIGURE 4. Kymograph record showing shell movements (1st and 3rd lines) and rates of pumping (2nd and 4th lines) of two oysters exposed to temperatures ranging from 24.0° to 24.5° C. from 10:10 A.M. to 4:15 P.M. Each vertical mark on the second line designates the discharge of 280 cc. of water pumped by the oysters, while each mark of the fourth line shows the discharge of 237 cc.

Incidentally, studies of many of our records indicate that often, at favorable temperatures, when the shells of the oysters are open and relatively motionless, large quantities of water are pumped. This is well demonstrated by the activities of the two oysters in Figure 4, one of which pumped the record quantities of water and the other also pumped at a rapid rate.

Almost immediately after passing the temperature level of 32.0° C. and, actually, in some instances, even between the temperatures of 31.1° and 32.0° C., certain signs began to appear, indicating that some oysters were being unfavorably affected. Among these signs were changes in the character of the shell movements, complete closing of the shells and cessation of pumping at frequent intervals, and a reduced rate of pumping even during the period when the shells remained open. Within the range of 34.1° to 36.0° C. these symptoms became more pronounced (Fig. 5).

Above 36.1° C. the type of shell movement became even more abnormal, and the oysters stayed closed approximately two-thirds of the time exposed.

To determine the significance of the differences in the average pumping rates of oysters at the different temperature intervals, a simple analysis of variance was employed. It was found unnecessary to compute all the *t*'s between adjacent temperature intervals. The same objective could be achieved by using a simple analysis of variance to test for homogeneity within each of the several apparently homogeneous levels, and also to test for the significance of the differences between these levels. These tests showed homogeneity within the following five temperature intervals: 4.1°–10.0° C., 10.1°–16.0° C., 16.1°–28.0° C., 28.1°–34.0° C., and 34.1°–38.0° C. The means for these five levels are shown in Figure 6 and are 593; 3,714; 8,727; 11,365 and 2,762 cc., respectively. The analysis indicated, as expected from inspection of this figure, that there were highly significant differences among these five levels. It was concluded, therefore, that the average pumping rate can be described as being related to these differences.

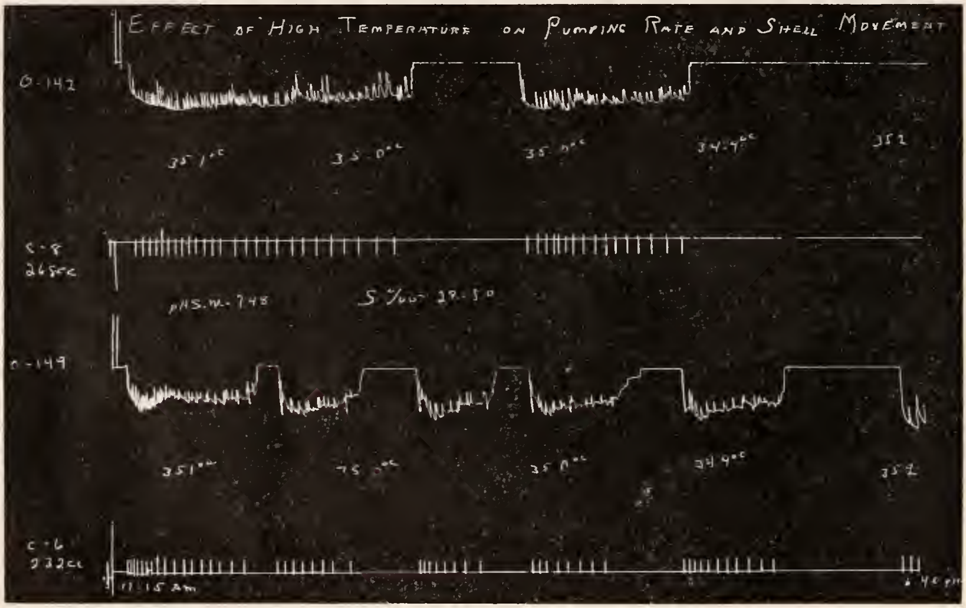


FIGURE 5. Kymograph record showing shell movements (1st and 3rd lines) and rates of pumping (2nd and 4th lines) of two oysters exposed to temperatures ranging from 34.9° to 35.2° C. from 11:15 A.M. to 6:40 P.M. Each vertical mark on the second line designates the discharge of 268 cc. of water pumped by the oysters, while each mark of the fourth line shows the discharge of 232 cc.

There are ecological situations in nature where changes in the temperature of the water are frequent and rapid. Many small, shallow, salt water ponds, of the type that can be adapted to shellfish cultivation, may belong to this category. Oysters living in such ponds or other similar bodies of water may experience such changes in temperature, especially during early spring and late autumn when the temperature of the water may quickly cool off during the night, decreasing to almost

0.0° C., and rapidly rise during the daytime. The question that naturally arises for such a situation is, how are the oysters affected by such changes and do they resume normal pumping regardless of the rapid change in the temperature from nearly freezing to about 15.0° C. or even higher?

The first series of experiments designed to answer the above question was conducted in October. The oysters were brought from outdoor tide-filled tanks, where the temperature of the water was about 13.0° C. One-half of the oysters, which served as the control, were placed in an aquarium in which the water temperature was kept at about 18.0° C. The other half were put in special containers in the refrigerator, where the temperature was about 3.0° C., and kept there from eight to 12 days. After this period at low temperature these oysters, as well as those serving as the control, were attached to kymographs. Extreme care was exercised during the handling to maintain the low temperature of the water for the refrigerated oysters. When the oysters of both groups were ready and attached to the kymographs a flow of water, about 18.0° to 20.0° C., was introduced simultaneously to all chambers, displacing the cold water surrounding the refrigerated oysters, and the behavior of all specimens was recorded from then on.

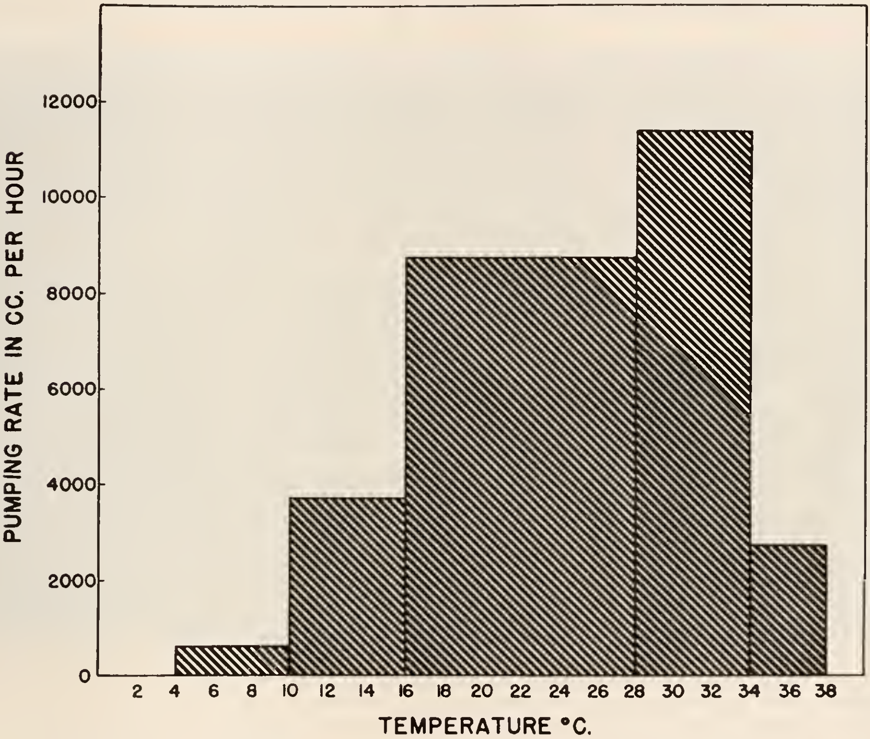


FIGURE 6. Mean rate of water pumping by oysters at five homogeneous temperature levels.

In a second series of experiments conducted in the middle of the winter (Fig. 7) the low-temperature oysters were not kept in the refrigerator but in the outdoor tanks where the temperature of the water was near 0.0° C. The oysters which were to serve as the control were, however, kept for three weeks in the laboratory at room temperature before being used in the experiments. In all other respects the methods used in the two series were the same. Altogether, in the two series of experiments, 33 control and 31 experimental oysters were used.

Student's *"t"* test was made to determine the significance of differences in the average pumping rate of the two groups. Because the "*t*'s" were far from significant, it was concluded that sudden upward changes in temperature, such as from 1.5° C. to about 18.0° C. (Fig. 7), had no significant effect on the pumping rate of the "cold water" oysters when such rate was compared with that of the control oysters. It was demonstrated, nevertheless, that there was a highly significant tendency of the oysters subjected to sudden changes from low to high temperature to open and pump almost immediately after the change was made, while the control oysters, as illustrated in Figure 7, usually required a much longer time to open their shells and begin pumping.

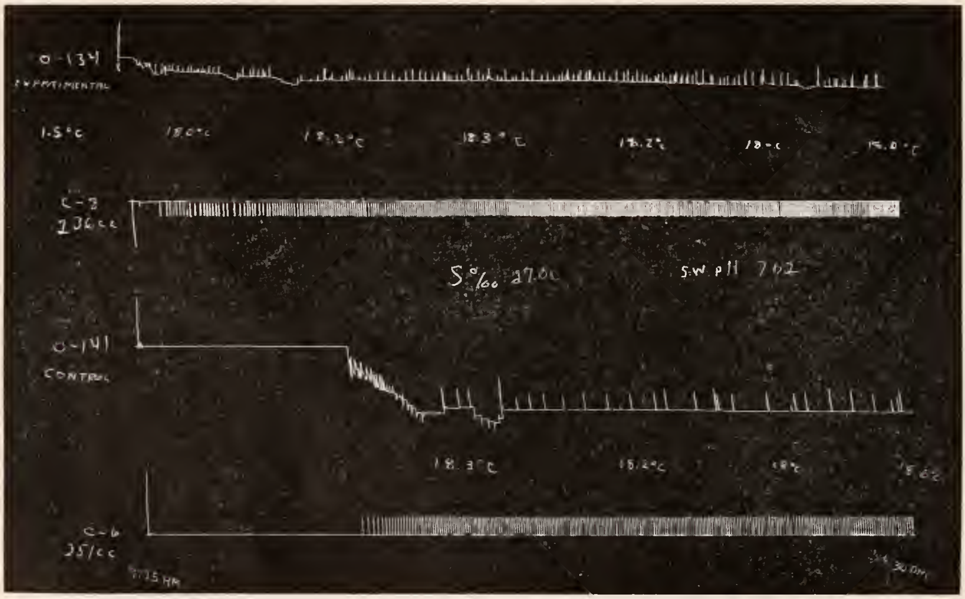


FIGURE 7. Kymograph record showing shell movements (1st and 3rd lines) and rates of pumping (2nd and 4th lines) of two oysters from 9:25 A.M. to 4:30 P.M. One, O-134, was exposed to rapid change of temperature from 1.5° to about 18.0° C., while the control oyster, O-141, was constantly subjected to the same temperature of about 18.0° C. Each vertical mark on the second line designates the discharge of 236 cc. of water pumped by the oysters, while each mark of the fourth line shows the discharge of 251 cc.

The results of these experiments differ from those of Galtsoff (1946) who, after keeping two oysters for 24 hours at 5.0° C. and then exposing them to a temperature of about 21.0° or 22.0° C., concluded that the shell movements of those oysters were abnormal and that they started to pump only on the third or fourth day after they had been placed in warmer water. We found no such abnormalities in the shell movements of the individuals used in our experiments (Fig. 7). Moreover, as already mentioned, the majority of our oysters previously kept in cold water opened and began pumping almost immediately, not after three or four days as found by Galtsoff. Our observations indicated, consequently, that the response of the oysters to a sharp and quick increase in temperature, from near 0.0° to about 15.0° to 20.0° C., is rapid and results in apparently normal pumping. The oysters, therefore, are physiologically well adapted to such changes which they, no doubt, often encounter in nature, for example, when living on the tidal flats where, at the autumn low tide, the night air may cool the water of the small pools containing the oysters almost to freezing, while the incoming tide may cover the same oysters with much warmer water.

Discussion

The rate of water pumping of different bivalves has been studied by a number of investigators. Among them, Jørgensen (1949) and Willemsen (1952) studied it in the common mussel, *Mytilus edulis;* Fox *et al.* (1937) and Rao (1953) worked on the closely related species, *M. californianus;* Willemsen (1952) also determined the quantities of water pumped by cockles, *Cardium edule;* Hopkins (1933, 1935) conducted extensive studies on the pumping of the Japanese commercial oyster, *Crassostrea gigas,* while Chipman and Hopkins (1954) determined the rate of water filtration by the common scallop, *Pecten irradians.* Most of these workers, except Hopkins, used the so-called indirect method based on a reduction of the number of particles or plankton organisms in suspension in the water in which the mollusks were kept. Hopkins used his own "cone" method by means of which he could determine only the relative rate at which the oysters pumped at different temperatures.

The most extensive observations on the rate of water transport were conducted on the American oyster, *C. virginica.* Except for Galtsoff's (1928a) pioneer experiments and Jørgensen's (1952) studies, investigators have used the direct or so-called "rubber apron" method, which permits measuring the actual quantities of water passed by oysters without interfering with their normal behavior. Most of these studies, however, including those of Loosanoff and Nomejko (1946), Loosanoff and Engle (1947) and Galtsoff *et al.* (1947), were made within relatively narrow temperature ranges not including the low or high temperature zones. Results of studies by Nelson (1935) and Loosanoff (1950), covering broader ranges, were presented only as brief abstracts.

As already mentioned, Galtsoff's (1928a) early experiments were criticized because the methods that he was then employing adversely affected the oysters (Nelson, 1938). Yet, regardless of the defects in his methods, Galtsoff, although giving rather low figures for the quantities of water pumped by oysters, indicated, nevertheless, quite correctly the general trend of the changes in the rate of pumping in relation to different temperature levels. For example, he observed that no feeding took place at 5.0° C. or lower. These conclusions, with the few exceptions demonstrated by our studies and by Hopkins' (1933) observations on the closely related species, still hold true for the majority of the population of *C. virginica* of northern waters. Galtsoff's observations that between 15.0° and 25.0° C. the fluctuations in the rate of pumping of individual oysters were small, strongly support the validity of our conclusions that between 16.0° and 28.0° C. the rate of pumping showed no marked fluctuation. Finally, his figures indicate a sharp drop in the rate of pumping after the temperature reaches and passes 34.0° C., as was also observed in our experiments.

Our observations showed that the highest average rate of flow of water through the gills of the oysters was achieved at about 29.0° C. (Table I). In this respect our results are in agreement with those of Nelson (1935) who determined that the maximum pumping in oysters occurs near 30.0° C. Our conclusions also resemble those of Galtsoff (1928b) that the optimum temperature for the mechanical activities of the oyster gills lies between 25.0° and 30.0° C. However, Galtsoff found that the maximum rate of pumping occurs at 25.0° C., somewhat lower than that recorded by Nelson (1935) or found in our studies. Nevertheless, considering Galtsoff's findings concerning the temperature at which the maximum activity of the oyster gills takes place, the three series of studies are in close agreement. Collier (1954) thinks, however, that even on the Gulf Coast the optimum temperature range for *C. virginica* lies between 15.0° and 25.0° C.

As to the maximum quantities of water pumped by *C. virginica,* there is considerable disagreement between the conclusions of the earlier workers, whose studies were reviewed by Galtsoff (1928a), and the more recent ones. The former estimated that oysters are capable of pumping only a few liters per hour. Wells (1926) gave the highest figure, advancing the opinion that at a favorable temperature oysters can average more than 7.5 liters per hour. His conclusions, however, were strongly challenged by Galtsoff (1928a), who expressed doubt that oysters can pump water at that rate.

Later investigators, who used the "rubber apron" method, have shown that the quantities of water pumped by oysters are much greater than those suggested by earlier workers. Nelson (1935) gives 26.0 liters per hour as the maximum quantity. Our maximum figure for a single oyster, as already mentioned, was 37,446 cc. for one complete hour, and an even higher rate was recorded for shorter periods. The maximum average hourly rate of pumping for a group of oysters was recorded within the temperature range of 28.1° to 30.0° C. and was 12,983 cc.; the maximum hourly rate, recorded between 30.1° and 32.0° C., was 16,253 cc. (Table I). Galtsoff *et al.* (1947) gave 9.6 liters as the average and 16.0 liters as the maximum hourly rate of water pumped by the oysters of York River, Va. Jørgensen's (1952) figures obtained by somewhat different methods are, nevertheless, very close to ours, being 11.0 and 15.5 liters per hour for the average and maximum rates, respectively. These figures attest the ability of the oysters to pump large quantities of water and indicate the importance of considering the water requirements of these mollusks in experimenting with them, or in their cultivation.

SUMMARY

1. Some adult Long Island Sound oysters are able to pump water at a temperature as low as about 1.0° C. Oysters with crystalline style and food in their stomachs may occasionally be found in northern waters in winter.

2. Approximately 15 per cent of the oysters exposed to temperatures ranging from 2.0° to 3.0° C., and approximately 50 per cent of the oysters kept between 3.0° and 4.0° C. formed pseudo feces.

3. The average and maximum rates of pumping of the groups of oysters exposed to temperatures from 0.0° to 38.0° C. were determined. The rate remained low

under 8.0° C. Within the range from 8.1° to 16.0° C. the rate steadily increased. Between that point and about 28.0° C. the rate showed no marked fluctuation. A further increase was noted between 28.1° and 32.0° C. It is within this range that the maximum average rate of pumping of 12,983 cc. per hour was recorded. Between 32.1° and 34.0° C. the rate was also rapid. Beyond 34.1° C. the oysters showed a marked decrease in the rate of pumping and their shell movements were abnormal.

4. The maximum rate of pumping for an individual oyster averaging 37,446 cc. per hour was recorded at the temperature of about 24.0° C. For short periods of five to 15 minutes the rate of pumping of the same oyster exceeded 40,000 cc. per hour.

5. Statistical tests showed homogeneity of the rates of pumping within the following five temperature intervals: 4.1°–10.0° C., 10.1°–16.0° C., 16.1°–28.0° C., 28.1°–34.0° C. and 34.1°–38.0° C. The means for these five intervals were 593; 3,714; 8,727; 11,365 and 2,762 cc. per hour, respectively. Highly significant differences among these five levels were indicated by statistical analysis.

6. The rate of pumping of oysters kept at a temperature below 5.0° C. and then quickly changed to the higher temperature of 18.0° to 20.0° C. was virtually the same as the control oysters, thus indicating that the response of the oysters to such changes in environment and their adjustment to these changes are rapid.

LITERATURE CITED

Chipman, W. A., and J. G. Hopkins, 1954. Water filtration by the bay scallop, *Pecten irradians,* as observed with the use of radioactive plankton. *Biol. Bull.,* **107**: 80–91.

Collier, A., 1954. A study of the response of oysters to temperature, and some long range ecological interpretations. *Nat. Shellfish. Assoc. Conv. Address,* 1953: 13–38.

Fox, D. L., H. N. Sverdrup and J. P. Cunningham, 1937. The rate of water propulsion by the California mussel. *Biol. Bull.,* **72**: 417–438.

Galtsoff, P. S., 1926. New methods to measure the rate of flow produced by the gills of oyster and other molluscs. *Science,* **63**: 233–234.

Galtsoff, P. S., 1928a. Experimental study of the function of the oyster gills and its bearing on the problems of oyster culture and sanitary control of the oyster industry. *Bull. Bur. Fish.,* **44**: 1–39.

Galtsoff, P. S., 1928b. The effect of temperature on the mechanical activity of the gills of the oyster (*Ostrea virginica* Gm.). *J. Gen. Physiol.,* **11**: 415–431.

Galtsoff, P. S., 1946. Reaction of oysters to chlorination. *U. S. Fish and Wildlife Service, Research Report,* **11**: 1–28.

Galtsoff, P. S., W. A. Chipman, Jr., J. B. Engle and H. N. Calderwood, 1947. Ecological and physiological studies of the effect of sulphate pulp mill wastes on oysters in the York River, Virginia. *Fish. Bull. 43, U. S. Fish and Wildlife Serv.,* **51**: 57–186.

Hopkins, A. E., 1933. Experiments on the feeding behavior of the oyster, *Ostrea gigas. J. Exp. Zool.,* **64**: 469–494.

Hopkins, A. E., 1935. Temperature optima in the feeding mechanism of the oyster, *Ostrea gigas. J. Exp. Zool.,* **71**: 195–208.

Jørgensen, C. B., 1949. The rate of feeding by *Mytilus* in different kinds of suspension. *J. Mar. Biol. Ass.,* **28**: 333–344.

Jørgensen, C. B., 1952. On the relation between water transport and food requirements in some marine filter-feeding invertebrates. *Biol. Bull.,* **103**: 356–363.

Jørgensen, C. B., 1955. Quantitative aspects of filter-feeding in invertebrates. *Biol. Rev.,* **30**: 391–454.

Loosanoff, V. L., 1950. Rate of water pumping and shell movements of oysters in relation to temperature. *Anat. Rec.,* **108**: 132.

LOOSANOFF, V. L., 1954. New advances in the study of bivalve larvae. *Amer. Sci.*, **42**: 607–624.

LOOSANOFF, V. L., AND H. C. DAVIS, 1950. Conditioning *V. mercenaria* for spawning in winter and breeding its larvae in the laboratory. *Biol. Bull.*, **98**: 60–65.

LOOSANOFF, V. L., AND J. B. ENGLE, 1947. Effect of different concentrations of micro-organisms on the feeding of oysters (*O. virginica*). *Fish. Bull. 42, U. S. Fish and Wildlife Serv.*, **51**: 31–57.

LOOSANOFF, V. L., AND C. A. NOMEJKO, 1946. Feeding of oysters in relation to tidal stages and to periods of light and darkness. *Biol. Bull.*, **90**: 244–264.

MOORE, H. F., 1910. Volumetric studies of the food and feeding of oysters. *Bull. U. S. Bur. Fish.*, **28**: 1295–1308.

NELSON, T. C., 1935. Water filtration by the oyster and a new hormone effect thereon. *Anat. Rec.*, **64**: 68.

NELSON, T. C., 1936. Water filtration by the oyster and a new hormone effect upon the rate of flow. *Proc. Soc. Exp. Biol.*, **34**: 189–190.

NELSON, T. C., 1938. The feeding mechanism of the oyster. I. *J. Morph.*, **63**: 1–61.

RAO, K. P., 1953. Rate of water propulsion in *Mytilus californianus* as a function of latitude. *Biol. Bull.*, **104**: 171–181.

VERWEY, J., 1952. On the ecology of distribution of cockle and mussel in the Dutch Waddensea, their role in sedimentation and the source of their food supply. *Arch. Néerl. de Zool.*, **10**: 172–239.

WELLS, W. F., 1926. Conclusions reached by the U. S. Public Health Service in experiments at Fisherman's Island. *Amer. J. of Pub. Health*, **16**: 10–12.

WILLEMSEN, J., 1952. Quantities of water pumped by mussels (*Mytilus edulis*) and cockles (*Cardium edule*). *Arch. Néerl. de Zool.*, **10**: 153–160.

QUANTITATIVE ASPECTS OF DEOXYRIBOSE NUCLEIC ACID (DNA) METABOLISM IN AN AMICRONUCLEATE STRAIN OF TETRAHYMENA[1]

BARBARA BROWN McDONALD[2,3]

Department of Zoology, Columbia University, New York 27, N. Y.

Of extreme cytological and genetic interest is the heteronucleate condition—the presence of both micro- and macronuclei—in most ciliate protozoans. The micronucleus is a permanent cell organelle, normally diploid, which divides mitotically during vegetative cell growth, and meiotically during conjugation or autogamy. On the other hand, the macronucleus, which contains far more chromatin as indicated by its size and staining capacity, appears to pull apart amitotically during vegetative growth; during conjugation or autogamy, it degenerates. Following this phenomenon, the new macronucleus develops from a division product of the synkaryon, presumably as a result of endomitosis (as demonstrated by Grell, 1953a, in a suctorean, *Ephelota gemmipara*).

Such obvious differences between these two types of nuclei led to the earlier belief that they contained two types of chromatin—idiochromatin in the micronucleus, and trophochromatin in the macronucleus (as noted by Wichterman, 1953). By a cytochemical study of vegetative individuals of *Paramecium caudatum,* however, Moses (1950) found that, although the macronucleus contains perhaps 40 times as much nucleoprotein as the micronucleus, both types of nuclei contain very similar *relative* amounts of deoxyribose nucleic acid (DNA), ribose nucleic acid and protein, which would seem to indicate that both are metabolically active. More recently, however, a difference in protein composition has been reported by Alfert and Goldstein (1955) for another ciliate, *Tetrahymena pyriformis* (mating types I and II, Elliott). Using a recently developed, direct staining method for basic protein (Alfert and Geschwind, 1953), they found the ratio of basic protein: DNA in the micronucleus to be about 1.7 times greater than that in the macronucleus.

As a result of its large amount of chromatin, the macronucleus might be expected to have more genetic influence than the micronucleus. This has, indeed, been found by Sonneborn (1947) to be the case in *Paramecium aurelia.* Organisms with a micronucleus of one genotype and a macronucleus of another (resulting from regeneration, after conjugation, of a part of the old macronucleus) show the characteristics carried by the macronucleus. He has referred to the micronucleus as the germinal nucleus, and the macronucleus as the somatic nucleus. More recently, Sonneborn (1954) has clearly demonstrated that genes in the micronucleus may be completely inactive. Phenotypic expression following conjugation in ciliates is, in fact, most complex. In *Paramecium* and *Tetrahymena,* organisms of identical genotype may differ as to phenotype (for example, mating type—Sonneborn, 1939; Nanney and Caughey, 1953). In some cases, differentiation appears to be influenced by the cytoplasm, which of course had been influenced by the pre-conjugation macronucleus (Sonneborn, 1951, 1953; Nanney 1953a, 1956, 1957). It appears that location in the cytoplasm determines which division products of a synkaryon will be micro- and which will be macronuclei, in both *Paramecium* (Sonneborn, 1953) and *Tetrahymena* (Nanney, 1953b).

---

[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Faculty of Pure Science, Columbia University.

[2] During part of this investigation the author was supported by a fellowship from The McCallum Foundation.

[3] Present address: Department of Biology, Dickinson College, Carlisle, Pennsylvania.

As exceptions to the rule that ciliates normally contain both types of nuclei, races and individuals occur in which micronuclei are absent. Such amicronucleate organisms, of course, cannot undergo autogamy, although they may conjugate with normal organisms (Chen, 1940; Sonneborn, 1953), and receive from them half a set of micronuclear chromosomes. Some amicronucleate strains are capable of vegetative growth for extended periods of time. Experimental strains of *Tetrahymena pyriformis,* for the most part, have no micronuclei (Corliss, 1953). In contrast, amacronucleate strains of ciliates have not been reported. Seshachar and Dass (1953) have observed in a peritrich, *Epistylis articulata,* that some daughter cells, receiving no macronucleus but only a micronucleus as a result of abnormal fission, eventually regenerated a macronucleus from a division product of the micronucleus. These various circumstances indicate that whereas the macronucleus is essential to the organism, the micronucleus is not. In amicronucleate strains, the macronucleus rather than the micronucleus is necessarily a permanent cell organelle.

In the present experiments, quantitative aspects of DNA metabolism in the macronucleus have been investigated in an amicronucleate strain of *Tetrahymena pyriformis*—strain H, isolated by Hetherington in 1930 (1933). Having no micronucleus, this strain has not been observed to undergo conjugation or autogamy, with the attendant degeneration and subsequent reformation of the macronucleus.

By means of microspectrophotometric analysis, the amount of DNA (indicated by the uptake of Feulgen dye, which has been shown to be proportional to the total DNA—Swift, 1953) has been determined at different stages of growth. The answers to a number of questions have been sought. For example, in the apparent absence of a mitotic apparatus, does the macronucleus divide equally between daughter cells? Before the next division, is the DNA precisely duplicated, as in mitotically dividing cells (Swift, 1950), or does an indefinite amount of synthesis occur? Does the DNA remain stationary in cells of a mass culture which has reached maximum growth? Finally, during what part of the growth cycle does the macronucleus synthesize DNA?

METHODS

I. *Stock cultures*

The stock of *Tetrahymena pyriformis* H used in these experiments was obtained from Mr. Sheldon Greer of the Department of Zoology, Columbia University, who in turn had obtained it from Dr. Seymour Hutner of the Haskins Laboratories, New York. The cells were cultured in 5-ml. amounts of 2% proteose peptone broth (Difco) in 16 × 150 mm. Pyrex test tubes. The tubes were arranged in a slanting position in a 25° C. constant temperature room. Stock cultures were transferred weekly (0.1-ml. inocula), at which time samples were spread on proteose peptone agar to test for possible contamination.

Cultures for experimental purposes were inoculated on the day before they were to be used, to insure that the cells would be in the logarithmic stage of growth.

II. *Fixation and staining of mass cultures*

Cells from a culture growing in a test tube were fixed directly on a chemically clean microscope slide, which was first placed in a paraffin-coated paper box (80 mm. long, 26 mm. wide, 12 mm. deep). After 10 ml. fixative (9 parts absolute alcohol: 1 part glacial acetic acid) were added, 0.25-0.5 ml. of the culture was delivered slowly from a fine-tipped pipette over the slide. In 10 minutes the slide was removed, drained, and held on its side against a paper towel while absolute alcohol was delivered slowly along the other edge from a fine-tipped pipette. The slide was then placed in absolute alcohol. After 10 minutes or longer, the slide was drained, 0.5% celloidin was run over the surface to which the cells were attached, and the slide was again drained and placed in 80% alcohol (modified from Chen, 1944). Throughout the subsequent treatment of the slide, precautions were taken so that the celloidin would not become dry and thus loosened from the slide.

Nuclei were stained according to the Feulgen procedure essentially as described by Di Stefano (1948) except that they were hydrolyzed in 2 N hydrochloric acid at 40° C. instead of in 1 N hydrochloric acid at 60°. This modification was used so that the time of hydrolysis would be less critical, since cells on different slides were to be compared. Photometric measurements indicated 55 minutes to be the optimal hydrolysis time.

After treatment with Feulgen preparation and bleach, the slide was rinsed during 5 minutes in 5 changes of water. The slide was run up through the alcohols to 1 part ether: 1 part absolute alcohol (which removed the celloidin), through absolute alcohol and xylol, and finally was mounted in oil of refractive index 1.572, which closely matched the refractive index of the cells.

III. *Growth of individual cells*

In a set of experiments to determine the generation times in small clones, individual cells were isolated under sterile conditions to hanging drops of proteose peptone broth. Growth was followed by observations every 15 minutes through a dissecting microscope.

Although the hanging drop method was quite satisfactory for following growth, it was not practical if the cells were to be fixed; when the cover slip was removed, the small drop of broth dried up very quickly. A different method was used to grow the cells in larger drops of medium (about 0.02 ml.) in small watch glasses (U. S. Bureau of Plant Industry Model, A. H. Thomas). These dishes (coated with silicone from Dow-Corning Sight Savers before sterilization to prevent the drops from spreading) were kept in the moist chamber of a petri plate lined top and bottom with wet filter paper in which holes had been cut for viewing the drops. To prevent fogging of the inner surface of the petri top, it was treated with a paste (Clersite, Chicago, Ill.) applied with sterile Wipettes.

For convenience, individual cultures were grown in the laboratory, at temperatures varying from 24–27°, averaging 25° C.

IV. *Fixation and staining of individual cells*

Individual containers for the fixation of cells from small clones were prepared by sealing paraffin rings to slides. The rings were formed by dipping a silicone-coated vial (10 mm. outside diameter), filled with cold water, into molten paraffin, and then into cold water. The paraffin coat was cut into rings about 5 mm. deep, which were slipped off the vial and sealed to the slide by careful heating. Two were placed on each slide, directly above notched circles which had been drawn on the opposite side of the slide with a diamond pencil. The notches, facilitating the location of cells, could be seen at the inner edges of the paraffin rings.

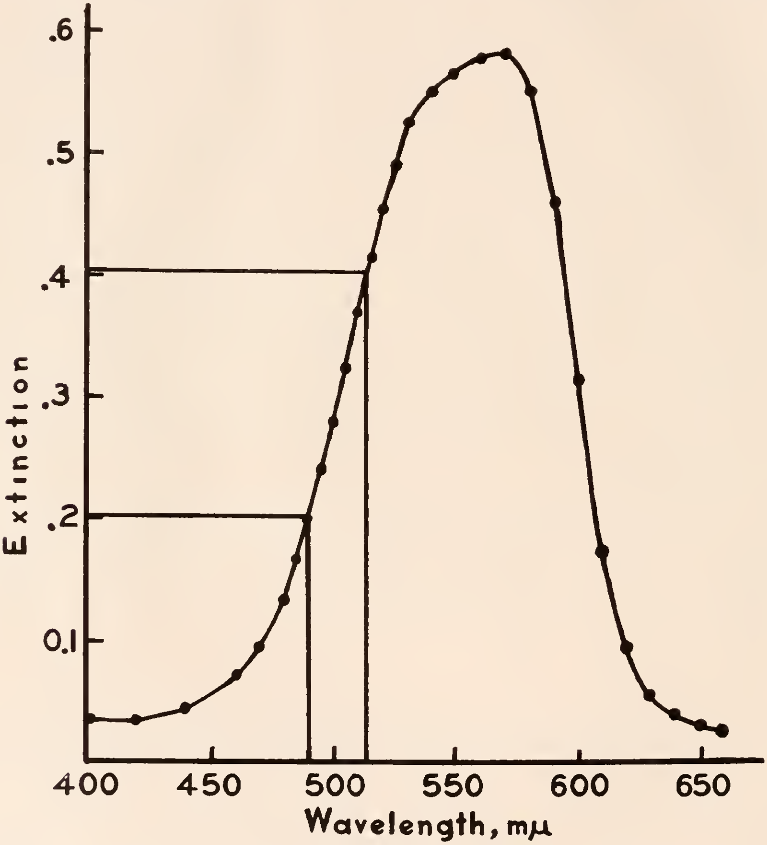


FIGURE 1. Absorption curve of a *Tetrahymena* macronucleus stained with Feulgen dye. The two wave-lengths (490 mμ and 514 mμ) used in making photometric measurements are indicated.

Fixation was carried out under two dissecting microscopes—one for the isolation of the cell, and one for its fixation. About 0.4 ml. of fixative was placed in one of the paraffin containers. The cell was delivered carefully from a micropipette at the surface of the slide, and a coverslip was placed on top of the container to minimize evaporation which would cause agitation of the fixative. After 10 minutes the fixative was removed with a pipette (whose fine tip was drawn out at an angle), and absolute alcohol was then carefully added. Usually this procedure resulted in sticking the cell to the slide; occasionally the cell became loose, in which case the alcohol was removed, and fresh alcohol was added. In this way the majority of cells could be saved.

After cells had been fixed in both containers on a slide, notations were made of the cells' locations and the slide was placed in a petri plate containing absolute alcohol. Within an hour the paraffin rings became loosened and were removed with a forceps. At this time the presence of the cells was checked under a dissecting microscope. The slide was rinsed in xylol-alcohol to remove any adhering paraffin molecules, in absolute alcohol, and then celloidin was run over its surface as previously described. Subsequent treatment of the slide differed from that already described only in that the celloidin was removed, after staining, by placing the slide in a petri plate containing ether-alcohol, when the presence of the cells was again checked under the microscope.

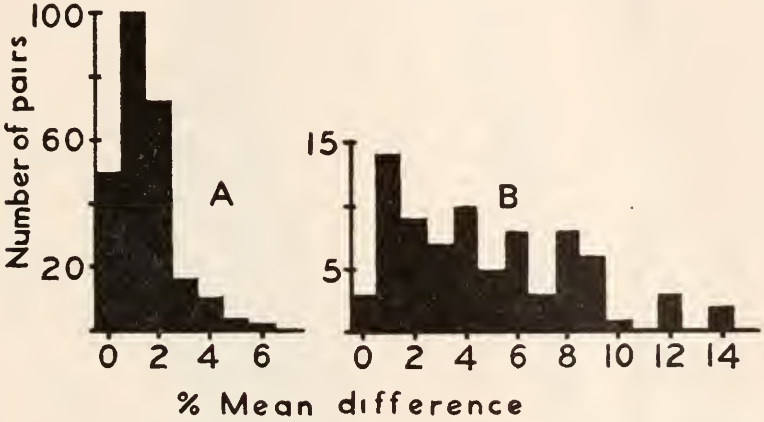


FIGURE 2. Variability of duplicate DNA determinations compared with variability of DNA content in sister cells. (A) % Mean difference in 263 pairs of duplicate DNA determinations. Range 0.0 to 5.7%, mean 1.4%. (B) % Mean difference of DNA values in 83 pairs of sister cells. Range 0.1 to 14.2%, mean 4.7%.

V. *Photometric methods*

Measurements were made with the microspectrophotometric apparatus described by Pollister (1952), using a Bausch & Lomb monochromator. Because it was desired to measure all the small clones fixed, and many of the nuclei were not spherical, the two-wave-length method described by Ornstein (1952) and Patau (1952) was used. Figure 1 shows a typical absorption curve for a Feulgen-stained nucleus. The two wave-lengths selected were 490 mμ and 514 mμ (which were also used by Patau). For a single DNA determination, two readings through both nucleus and background were made at each wave-length. Two determinations were made and averaged for each cell from a small clone; between determinations, the fine adjustment and the condenser of the microscope were refocused, and the phototube diaphragm was readjusted. The % mean difference of duplicate determinations on 263 cells ranged from 0 to 5.7, with an average of 1.4 (Fig. 2A).

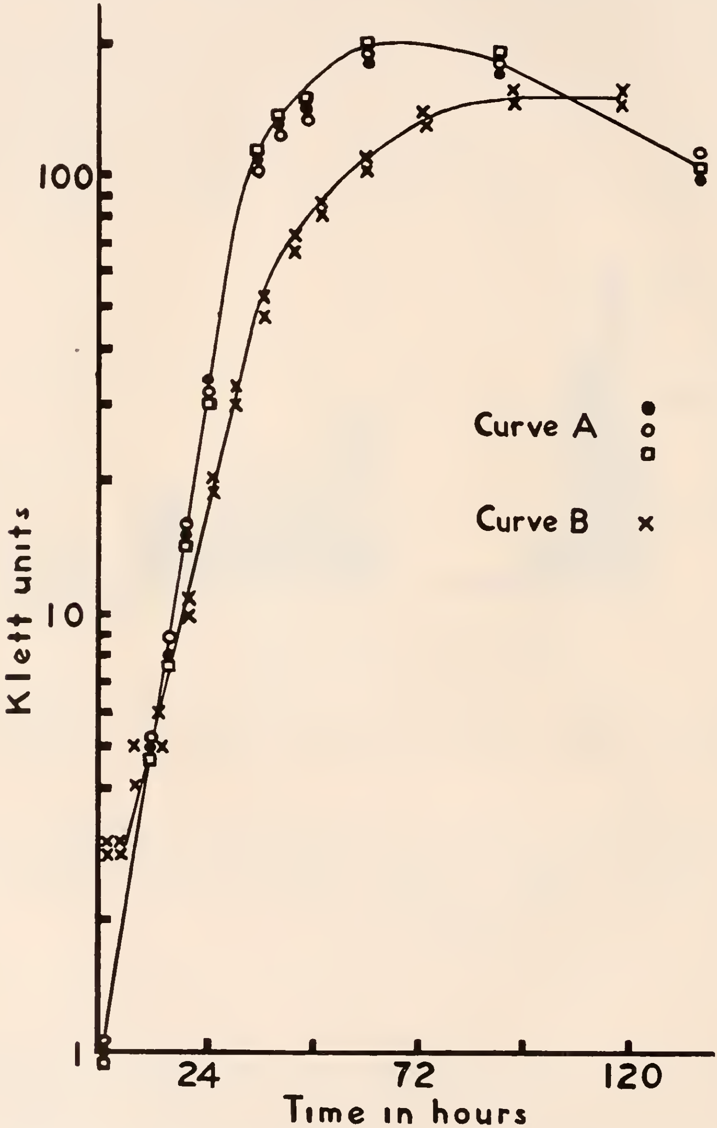


Figure 3. Growth curves for *Tetrahymena pyriformis* H. Both curves are drawn from turbidity readings in a Klett photoelectric colorimeter. Curve A is based on three tubes of cells grown in a roller. Curve B is based on a series of tubes, slanted but not otherwise aerated. Maximum growth for both A and B was about 3×10^5 cells per ml. (Although all the growth tubes were the same size, two different sizes of tubes were used for the two sets of readings, accounting for the different heights of the curves.)

I. *Growth of Tetrahymena*

The ability of *Tetrahymena* to thrive under controlled conditions in non-particulate, sterile medium makes it an ideal protozoan for biological research. Although proteose peptone broth was used in this investigation, *Tetrahymena* can be grown also in media of carefully measured, known constituents (Elliott and Hayes, 1953; Kidder, Dewey and Heinrich, 1954). The growth response in both proteose peptone and defined media is quite reproducible.

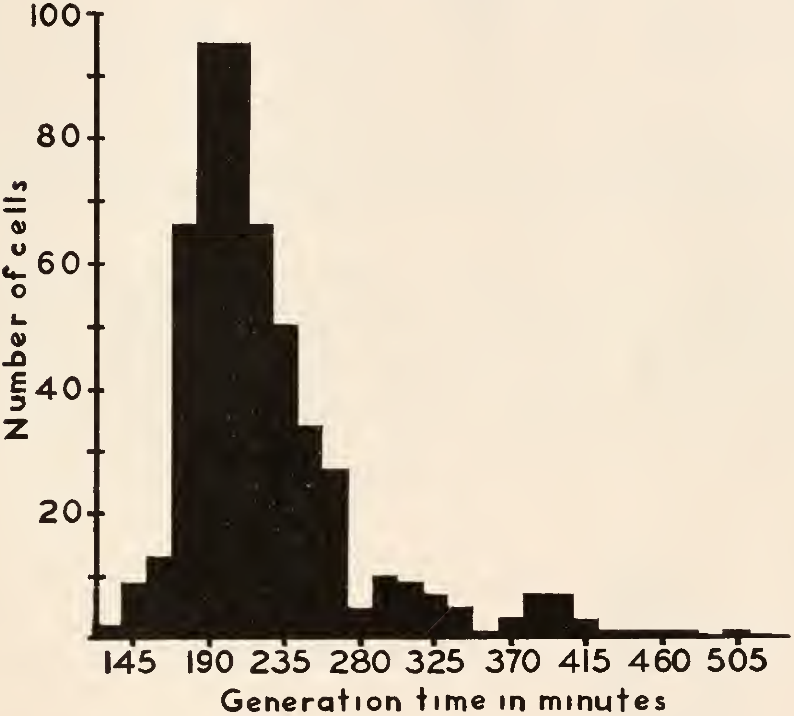


Figure 4. Generation times in isolated clones. A total of 515 cells (160 two-cell clones and 195 individual cells) are represented. Data for the different groups of cells included in this histogram are given in Table I.

A. *Growth in mass cultures. Tetrahymena* shows a pattern of growth similar to that for other micro-organisms—a short lag period (when the inoculum is from a culture in stationary phase); a logarithmic stage of growth, during which the cell number doubles in regular time intervals; a period of deceleration, when the rate of cell divisions decreases; and a stationary period, during which the number of cells remains constant, with few cell divisions or deaths.

In Figure 3 are shown two different growth curves for cells grown in 2% proteose peptone broth, based on turbidity readings in a Klett photoelectric colorimeter. Curve A represents the growth response of three tubes of cells (inocula

TABLE I

Generation times of individual cells

Growth conditions	Number of cells	Range (min.)	Mean ± S.D. (min.)	Mode (min.)
Hanging drops	170 (includes 84 2-cell clones)	135–505	215 ± 53.7	190–205
Drops in watch glasses	152 (76 2-cell clones)	150–465	220 ± 58.1	190
Drops in watch glasses*	90	155–480	240 ± 60.2	205
Drops in watch glasses*	103	150–400	227 ± 43.7	205
Total	515	135–505	223 ± 53.0	190–205

* These generation times were observed in the course of studying DNA synthesis (see Results, IIB3). Photometric measurements were made of the 90 cells in line 3; the 103 cells in line 4, which received identical treatment, were not measured (because of cell loss, particles overlying a nucleus, or a broken nucleus).

from a log phase culture) aerated in a roller. The generation time during log phase is seen to be about four hours. Curve B shows the response of cells (inocula from stationary phase) grown undisturbed in slanted tubes but not otherwise aerated—the conditions under which stock and mass experimental cultures were grown in the present experiments. The two readings at each time period of Curve B represent two tubes of a series inoculated at the same time; after a tube was shaken to distribute the cells, and a reading was made, it was discarded. Curve

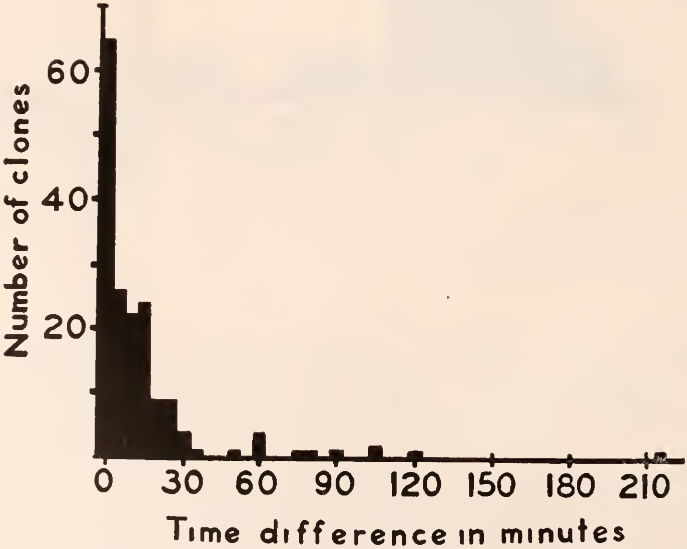


FIGURE 5. Differences in generation times between sisters in 174 two-cell clones.

B differs noticeably from Curve A in the long generation time of 7 hours and the extended period of deceleration. These conditions undoubtedly resulted from the limited availability of oxygen. Both curves clearly indicate the reproducibility of growth response of *Tetrahymena* in 2% proteose peptone broth under controlled conditions.

B. *Growth in small clones.* Single cells which had been isolated from cultures in log phase to either hanging drops or drops in small watch glasses were timed through two divisions. Generations were calculated between the times the sisters actually separated, to the nearest 5-minute intervals. The distribution of generation times is shown in Figure 4, and the separate experiments are summarized in Table I. The over-all range is 2 hours 15 minutes (135 minutes) to 8 hours 25 minutes (505 minutes); the mean generation time is 223 ± 53.0 minutes. This is close to the value of 4 hours (240 minutes) computed from Klett readings of cultures grown in tubes which were aerated on a roller, indicating that the conditions in small drops were favorable for rapid growth.

C. *Generation times of sister cells.* The differences in generation times of sister cells are shown in Figure 5. In contrast to the wide range of generation times among different clones, sisters tend to have very similar generation times, the mean difference being 14 minutes. Rarely did a cell divide more than 30 minutes later than its sister, and often the sisters divided simultaneously (*i.e.,* within the same two-minute interval). The difference between sisters was not correlated with length of generation time. In some clones the two sisters divided almost synchronously after 5 to 7 hours, while some cells with short generation times had sisters which did not divide until an hour or more later.[4]

II. *Nuclear phenomena in the life cycle*

A. *Cytology.* Photographs of several cells from a mass culture in log phase, stained by the Feulgen method and counterstained with fast green, are shown in Figure 6. Macronuclei of *T. pyriformis* H range in diameter from about 5 to 12 μ, with an average size of about 8 μ. Before division starts, the enlarged nucleus appears to consist of strands of chromatin granules wound into a ball (stage 1). The nucleus then elongates, and the strands are pulled out along its length (stage 2). As division proceeds, and the nucleus separates into the two daughters (stage 3), broken ends of strands can sometimes be seen. Part of a strand (or strands) may remain behind in the cytoplasm (stage 4) where it condenses into a small Feulgen-positive body. Similar in appearance to micronuclei, these bodies are not present in all cells, and in time seem to be resorbed by the cytoplasm. One of these particles is visible in the left of the two interphase cells (stage 5). The relatively homogeneous interphase nucleus appears to be made up of fine threads of chromatin.

---

[4] Separation of sisters and "conditioning" of medium did not seem to affect generation time. About 12 hours after 9 individuals had been isolated to drops of broth in watch glasses, all but one of the cells in each drop resulting from the interim divisions were removed. An hour after these cells had divided, one sister of each pair was placed in a fresh drop of medium. All the pairs of separated sisters divided within 20 minutes of each other—three divided synchronously, five cells in fresh medium divided sooner than their sisters, and one divided later.

In these photographs, cytoplasmic vacuoles are demonstrated by the fast green stain, but the mouths of the organisms are not clearly distinguishable.

B. *Photometric analysis of DNA.* 1. *Amount of DNA per cell in mass cultures.* A sample of cells from a logarithmically growing culture (I) (24 hours after inocu-

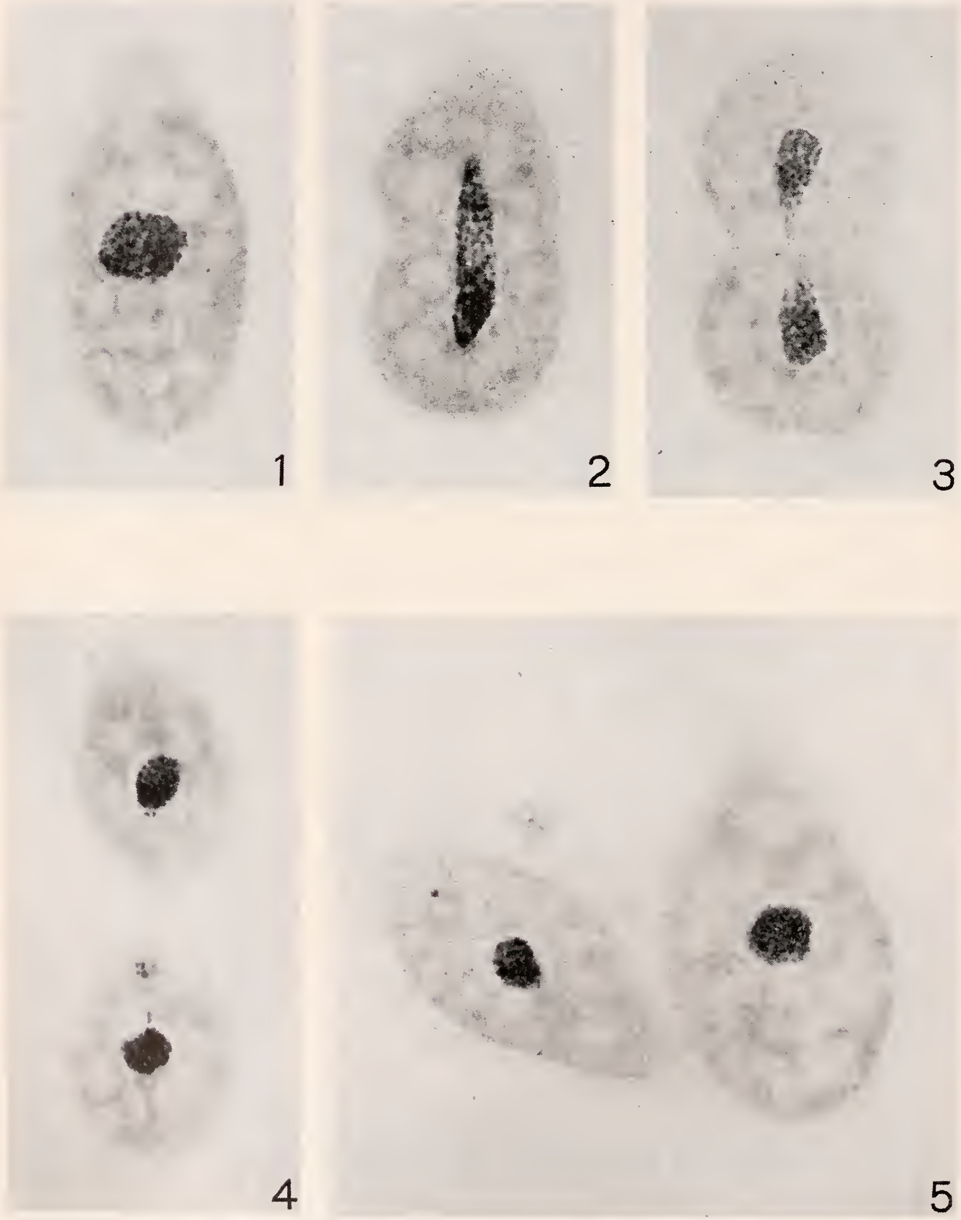


FIGURE 6. Photographs of *Tetrahymena pyriformis* H stained with Feulgen and fast green dyes. Dividing cells in different stages are shown in 1, 2, 3, and 4, and interphase cells in 5. See text for further explanation. Magnification about 1050 ×.

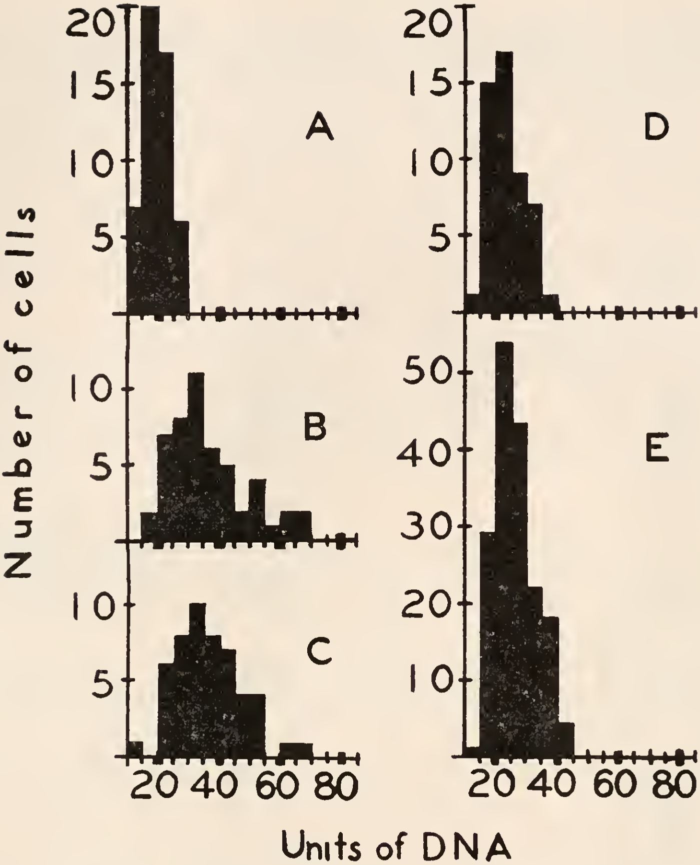


FIGURE 7. Distribution of DNA in mass cultures and isolated cells. (A) Culture I, one day old (log phase). Amount of DNA in 50 dividing daughters (each from a different pair). Range 12.5 to 29.2; mean 19.3 ± 4.35 S. D.; mode 17. (B) Culture I, one week old (stationary phase). Amount of DNA in 50 cells. Range 17.9 to 68.0; mean 35.9 ± 12.9 S. D.; mode 32. (C) Culture I, two weeks old. Amount of DNA in 50 cells. Range 13.9 to 66.6; mean 36.0 ± 11.0 S. D.; mode 32. (D) Culture II, one day old (log phase) (subculture of one-week old Culture I). Amount of DNA in 50 dividing daughters (each from a different pair). Range 13.7 to 36.8; mean 22.8 ± 5.81 S. D.; mode 22. (E) Isolated dividing daughters. Amount of DNA in 173 cells (83 pairs and 7 individuals). Range 12.9 to 43.2; mean 25.7 ± 6.15 S. D.; mode 22.

lation) was fixed and stained. One daughter nucleus of each of 50 dividing pairs (similar to stage 4, Fig. 6) was measured; presumably these values would indicate the basic, or minimal, amount of DNA. (Since the cytoplasm was invisible, criteria for dividing daughters were shape and appearance of nuclei—two rather tear-shaped and granular-looking nuclei, usually with part of a strand of chromatin extending from one toward the other.) One week later this culture, in stationary phase, was sampled again, and 50 nuclei were measured. At the same time, a subculture (II) was inoculated from Culture I. Twenty-four hours later Culture II was sampled, and 50 of its dividing daughters were measured. When Culture I was two weeks old it was sampled again, and 50 more nuclei were measured.

The histograms for the values of DNA in arbitrary units are shown in Figure 7. The amounts in daughter nuclei of Culture I at log phase (A) ranged from 12.5 to 29.2, with a mean of 19.3 (S. D. 4.35); those of Culture II (D) ranged from 13.7 to 36.8, with a mean of 22.8 (S.D. 5.81). The one-week-(B) and two-week-(C) old cells from Culture I contained amounts of DNA ranging from 17.9 to 68.0 units, and 13.9 to 66.6 units, respectively, the week-old stationary culture having a mean of 35.9 (S. D. 12.9), and the two-week-old culture having a mean of 36.0 (S. D. 11.0). From these values it appears that most non-dividing cells in stationary phase have doubled their DNA. The lowest values might conceivably represent cells which had recently divided (although no dividing cells were seen in these samples), or cells which were dying or dead.

2. *Comparison of DNA in sister cells.* Photometric measurements of DNA in pairs of newly separated sister nuclei indicate that, in general, division is quite even. Among 83 pairs of sisters, the % mean difference ranged from 0.1 to 14.2, with an average of 4.7 (Fig. 2B). As mentioned previously (Methods, V), the instrumental error averaged 1.4% (Fig. 2A), approximately ⅓ of the difference between sisters.

3. *DNA in cells in small clones.* An attempt to investigate the time of DNA synthesis during cell growth has been made using the information described above: (1) that sister cells generally have very similar generation times; (2) that distribution of DNA between sister cells is fairly equal; and (3) that following a division, DNA appears to be approximately doubled.[5]

Figure 8 summarizes the plan devised for this investigation, from which data are available for 90 clones. The time of the division of an isolated cell (C) was noted, and at some time after that one of the resulting sisters (C_1) was fixed. When the other sister (C_2) had nearly completed its subsequent division it, in turn, was fixed. In view of the previous data indicating the close similarity of generation times between sister cells, it was assumed that the generation time of C_2 gave a fairly reliable measure of the potential generation time of C_1.[6] Photometric measurements were made of all three macronuclei—that of interphase cell C_1, and those of the two daughters of C_2 (designated C_{2a} and C_{2b}). Adding the DNA in C_{2a} and C_{2b} for the total amount in C_2 indicated the amount of DNA which C_1 would have contained by the time it reached its next division. The ratio of C_1/C_2, then, indicates the relative amount of DNA in C_1 when it was fixed.

---

[5] Some additional evidence suggesting the duplication of DNA comes from seven pairs of synchronously dividing sisters, each of whose four daughter nuclei were measured photometrically. Among four of these pairs, the two daughter mates with the lower total amount of DNA had 94%, 96%, 97%, and 97% as much DNA as the two higher daughter mates. In the other three pairs, the daughters had become detached, so the mates could not be differentiated. The most extreme possible relative values would have been 82%, 89%, and 96%; the closest possible, 91%, 99%, and 100%.

[6] The generation time of C_2 was known quite closely, although, since dividing cells were fixed, the time of separation had to be estimated by appearance. Cells which are in the process of dividing become quiescent until the division is nearly completed, when they begin swimming, pulling, and twisting. Assuming the time spent dividing to last from the time a cell quiets down and a constriction begins to appear, to the time the daughters actually pull apart, it has been observed to take from about 20 to 35 minutes, regardless of the length of generation time. In these experiments, an average of 25 minutes was used as a basis for calculation. For these cells, the length of interphase time, then, is based on the generation time (known time of separation at the first division to the estimated time of separation at the second division) minus 25 minutes.

If the original cell C had divided its DNA precisely between C_1 and C_2, and no DNA had been synthesized when C_1 was fixed, one would obtain the ratio 0.5. On the other hand, if C_1 had duplicated its DNA by the time it was fixed, one would obtain the ratio 1.0. Ratios of 0.625, 0.75, and 0.875 would indicate, respectively, that 25%, 50%, and 75% of synthesis had occurred.

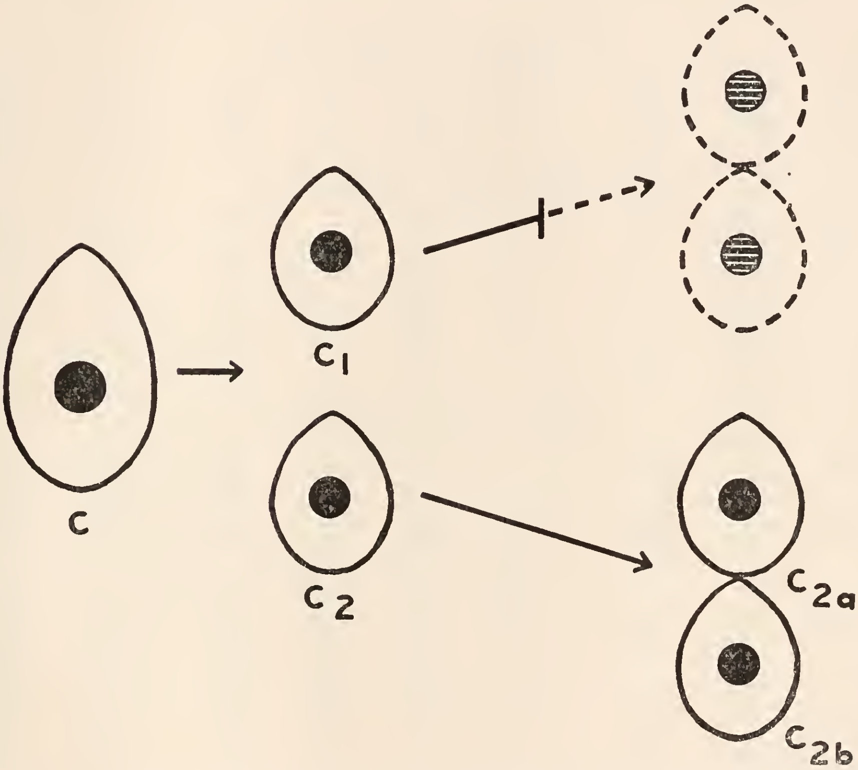


FIGURE 8. Scheme for fixing sister cells. The figure on the left represents an isolated cell (C), in time dividing into the two sister cells in the middle (C_1 and C_2). During the following interphase C_1 was fixed, as indicated by the vertical line, when its DNA might or might not have increased. C_2 was not fixed until its next division, as indicated at bottom right; its generation time was a measure of the potential generation time of its fixed sister, C_1. The total DNA in the C_2 daughters (C_{2a} and C_{2b}) represented the amount which C_1 would have contained at its next division.

In Table II the data are summarized, with the 90 clones grouped according to generation times, and each group arranged according to the age of C_1 when it was fixed. Also included are the period of interphase (see footnote 6) elapsing between the fixation of C_1 and the start of the C_2 division, the relative interphase age of C_1 (age of C_1/length of C_2 interphase), the amounts of DNA in the measured cells, and the relative DNA content of C_1 (C_1 DNA/C_2 DNA). Table I, line 3, summarizes the generation time data for the C_2 cells, and Figure 7E shows the distribution of DNA values for C_{2a} and C_{2b}.

TABLE II

Photometrically measured clones arranged according to generation times

Clone No.	Age C_1 (min.)	Time to next div. (min.)	Age C_1 / Interphase*	Amount of DNA (in arbitrary units)				C_1 DNA / C_2 DNA
				C_1	C_2	C_{2a}	C_{2b}	
Generation time 155–165 minutes								
1	75	60	0.56	44.8	44.3	21.8	22.5	1.01
2	95	35	.73	60.8	58.7	26.7	32.0	1.04
Generation time 170–180 minutes								
3	90	65	.58	53.0	38.6	18.0	20.6	1.37‡
4	95	50	.66	54.3	58.0	28.5	29.5	.94
5	125	25	.83	48.7	54.9	27.2	27.7	.89
6	130	25	.84	62.6	53.6	25.0	28.6	1.17
7	140	15	.90	49.2	49.6	22.7	26.9	.99
8	145	10	.94	51.9	49.0	24.2	24.8	1.06
Generation time 185–195 minutes								
9	30	140	.18	27.1	49.8	23.7	26.1	.54
10	35	130	.21	35.3	57.1	27.9	29.2	.62
11	35	125	.22	23.2	48.0	23.4	24.6	.49
12	50	120	.29	21.6	38.7	19.0	19.7	.56
13	50	110	.31	24.5	32.0†	16.0	—	.77
14	60	110	.35	33.7	34.5	16.5	18.0	.98§
15	65	105	.38	30.7	58.2	26.8	31.4	.53
16	75	85	.47	40.2	51.9	24.4	27.5	.78
17	75	85	.47	64.0	55.0	26.1	28.9	1.16
18	90	80	.53	50.5	45.2	21.0	24.2	1.12
19	120	45	.73	52.0	52.1	25.1	27.0	1.00
20	120	40	.75	47.1	52.1	25.2	26.9	.90
21	155	05	.97	41.4	47.9	22.9	25.0	.86
Generation time 200–210 minutes								
22	30	150	.17	32.1	60.0	29.9	30.1	.53
23	35	150	.19	26.6	43.7	20.6	23.1	.61
24	35	145	.20	27.8	43.4	21.6	21.8	.64
25	45	130	.26	28.0	35.5	17.7	17.8	.79§
26	65	115	.36	33.3	46.8	23.0	23.8	.71
27	90	90	.50	56.6	58.7	29.0	29.7	.97
28	90	85	.51	34.8	48.2†	24.1	—	.72
29	90	85	.51	32.1	40.6	19.6	21.0	.78
30	90	85	.51	36.8	37.3	18.1	19.2	.99
31	95	85	.53	42.6	78.6	38.8	39.8	.54§

* Interphase equals generation time minus 25 minutes.

† Estimate of C_2 DNA based on measurement of one daughter; other daughter unmeasurable.

‡ Unequal division of C obvious from C_1/C_2 ratio.

§ Unequal division of C presumed from exceptional amount of DNA in C_2, and unusual C_1/C_2 ratio for age of C_1.

TABLE II—*Continued*

Clone No.	Age C_1 (min.)	Time to next div. (min.)	Age C_1 / Interphase*	Amount of DNA (in arbitrary units)				C_1 DNA / C_2 DNA
				C_1	C_2	C_{2a}	C_{2b}	
Generation time 200–210 minutes—*Continued*								
32	100	85	.54	34.1	78.9	35.5	43.4	.43§
33	110	70	.61	60.9	63.6	31.5	32.1	.96
34	125	55	.69	53.8	62.6	31.1	31.5	.86
35	130	45	.74	48.8	49.3	23.8	25.5	.99
36	155	20	.89	34.2	33.1	15.7	17.4	1.08
37	160	15	.92	47.5	43.1	19.9	23.2	1.10
38	165	20	.89	40.4	34.4	16.4	18.0	1.17
Generation time 215–225 minutes								
39	70	130	.35	35.8	46.8	23.2	23.6	.77
40	95	100	.49	37.8	61.1	30.0	31.1	.62
41	150	45	.77	42.9	41.6	20.6	21.0	1.03
42	165	30	.85	49.7	47.3	21.7	25.6	1.10
43	165	25	.87	65.7	72.5	35.2	37.3	.91
44	170	30	.85	60.8	71.0	34.8	36.2	.86
45	180	10	.95	33.9	38.4	17.5	20.9	.88
46	185	15	.93	51.1	50.6†	25.3	—	1.01
Generation time 230–240 minutes								
47	35	180	.16	22.3	45.8	22.0	23.8	.49
48	60	150	.29	22.0	59.8†	29.9	—	.37‡
49	65	140	.32	35.6	54.4	25.6	28.8	.66
50	70	135	.34	45.2	40.5	19.4	21.1	1.12
51	140	70	.67	54.7	60.5	27.8	32.7	.91
52	155	55	.74	45.7	45.9	21.1	24.8	1.00
53	180	35	.84	62.7	59.4	29.2	30.2	1.05
54	175	30	.85	59.6	69.6	34.3	35.3	.86
Generation time 245–255 minutes								
55	30	190	.14	19.4	58.9	28.6	30.3	.34‡
56	35	195	.15	24.9	41.7	19.1	22.6	.60
57	60	160	.27	17.6	27.5	12.9	14.6	.64
58	130	90	.57	48.5	64.4	27.6	36.8	.75
59	145	85	.63	41.6	51.6†	25.8	—	.81
60	150	75	.67	43.8	43.8	21.0	22.8	1.00
61	155	65	.71	41.0	37.0	17.5	19.5	1.11
62	160	65	.71	45.7	50.4	25.1	25.3	.91
63	160	60	.73	36.4	47.7	23.5	24.2	.76
64	175	55	.76	46.2	47.8	22.5	25.3	.98
65	190	30	.86	41.0	34.7	17.2	17.5	1.18‡

TABLE II—*Continued*

Clone No.	Age C_1 (min.)	Time to next div. (min.)	Age C_1 / Interphase*	Amount of DNA (in arbitrary units)				C_1 DNA / C_2 DNA
				C_1	C_2	C_{2a}	C_{2b}	
Generation time 260–270 minutes								
66	115	130	.47	29.4	75.5	33.4	42.1	.39‡
67	120	115	.51	41.8	65.1	32.3	32.8	.64
68	145	90	.62	46.8	61.0	29.6	31.4	.77
69	150	85	.64	56.1	65.2	28.7	36.5	.86
70	150	85	.64	47.2	43.8†	21.9	—	1.08
71	155	90	.63	33.2	40.2	18.3	21.9	.83
72	155	80	.66	35.0	34.9	16.1	18.8	1.00
73	200	40	.83	50.0	58.9	28.4	30.5	.85
Generation time 275–285 minutes								
74	65	190	.26	35.3	52.8	24.9	27.9	.66
75	65	185	.26	41.4	69.7	34.8	34.9	.60
76	200	55	.79	42.1	52.0	22.8	29.2	.81
Generation time 290 minutes								
77	120	145	.45	40.0	82.0	38.8	43.2	.49§
Generation time 315 minutes								
78	220	70	.76	69.9	64.2†	32.1	—	1.09
Generation time 320–330 minutes								
79	120	180	.40	30.8	43.7	20.5	23.2	.71
80	135	170	.44	45.0	76.5	37.2	39.3	.59§
81	180	120	.60	48.3	47.6	23.2	24.4	1.01
Generation time 335–345 minutes								
82	150	165	.48	27.2	54.6	26.1	28.5	.50
83	245	70	.78	28.2	33.6	15.5	18.1	.84
Generation time 350 minutes								
84	90	235	.28	27.6	61.3	26.3	35.0	.45
Generation time 365–375 minutes								
85	90	250	.26	26.2	58.5	27.3	31.2	.45
86	180	160	.53	31.0	71.4	35.2	36.2	.43§
87	300	45	.87	35.3	40.5	19.4	21.1	.87

TABLE II—*Continued*

Clone No.	Age C_1 (min.)	Time to next div. (min.)	$\frac{\text{Age } C_1}{\text{Interphase}}$*	Amount of DNA (in arbitrary units)				$\frac{C_1 \text{ DNA}}{C_2 \text{ DNA}}$
				C_1	C_2	C_{2a}	C_{2b}	
Generation time 385 minutes								
88	35	325	.10	16.6	38.3	17.6	20.7	.43
Generation time 405 minutes								
89	35	345	.09	18.2	31.8	15.8	16.0	.57
Generation time 480 minutes								
90	300	155	.66	54.4	43.3	20.3	23.0	1.25‡

The deviations to be expected from the ideal C_1/C_2 ratios of 0.5 and 1.0, before and after synthesis, have been calculated from the DNA values of C_{2a} and C_{2b} nuclei, on the assumption that the parent cells (C) may have divided with the same degree of equality as did their daughters (C_2) (Table III). For these calculations, four ratios were used:

(1) low daughter/high daughter × 2 (the situation to be found if the interphase cell contained the lower amount of DNA and had not started synthesis);
(2) high daughter/low daughter × 2 (if the interphase cell contained the higher amount of DNA and had not started synthesis);
(3) low daughter/high daughter (if the interphase cell contained the lower amount of DNA and had completed synthesis); and
(4) high daughter/low daughter (if the interphase cell contained the higher amount of DNA and had completed synthesis).

Obviously any C_1/C_2 ratios of 0.42 or less, or 1.18 or more, must have resulted from divisions approximately as unequal as the 13 most unequal cases listed in Table III. Table II shows three such low and three such high ratios (marked by symbol ‡). In three of these clones (Nos. 3, 65, 66) the C_2 DNA was considerably above or below the mean (51.4 units) for this group of cells. Partial synthesis, obviously, would tend to obscure other such unequal pairs, and asynchronous growth probably would also be reflected by unusual C_1/C_2 ratios. In 7 cases (marked in Table II with symbol §), however, inequality of DNA content is suggested by exceptionally high or low C_2 values, combined with C_1/C_2 ratios unusual for the age of C_1. A total of 13 presumably unequal pairs among the 90 clones are thus designated—which, remarkably, is also the number of the most unequal of the 83 C_{2a}–C_{2b} cells listed in Table III.

From the remainder of Table III it appears that for the bulk of clones, C_1/C_2 ratios of 0.59 or less indicate that C_1 had synthesized little or no DNA, whereas ratios of 0.85 or more indicate that when C_1 was fixed it probably had completed duplication of its DNA. Based on these figures, the data from Table II have been summarized in Table IV according to (A) the age of C_1, (B) the interphase time elapsing from the fixation of C_1 to the start of the C_2 division, and (C) the relative interphase age of C_1. In the more detailed examination of the clones, below, the numbers refer to Table II.

Consideration of the early part of interphase shows that, of 11 clones in which the C_1 cells were fixed within the first 30–35 minutes, 7 have C_1/C_2 ratios of less than 0.59, indicating that probably no synthesis had occurred. Ratios for the other four clones (Nos. 10, 23, 24 and 56) are all in the low 0.60's. At the other end of interphase, of 40 clones in which C_1 was fixed 80 minutes or less before the next division began, 37 have C_1/C_2 ratios of 0.85 or more, indicating that in these cases synthesis probably had been completed. Two of the three exceptional clones (Nos. 76 and 83) had C_1/C_2 ratios which were only slightly low (0.81 and 0.84). The other clone, No. 63, had a ratio of 0.76; its C_2 value, however, was not unusually high, which suggests the possibility that asynchronous growth of the sister cells had occurred.

Despite the wide range of interphase times among individual cells, it thus appears that DNA synthesis generally does not begin for a period of perhaps a half hour after the previous division, and is completed a considerable length of time—about 80 minutes—before the next division begins. The process of synthesis, then, would occupy a fairly short period of time in cells with short generation times, and a relatively longer period in cells with long generation times. Presumably C_1 cells fixed during this interim would be in the process of synthesizing DNA. Among 39 such clones, 18 do indeed have C_1/C_2 ratios which indicate that partial synthesis had occurred. Nine others have ratios above 0.85, suggesting that synthesis might have been completed when C_1 was fixed. The other 12 have ratios under 0.59, which appear to indicate that no synthesis had occurred. Six of these clones (Nos. 31, 32, 66, 77, 80, and 86), however, had such high C_2 values as to suggest that

TABLE III

Expected C_1/C_2 ratios of DNA at the beginning (before synthesis) and end (after synthesis) of the interphase period, based upon the distribution of the DNA of C_2 between its daughter cells (C_{2a} and C_{2b})

No. of pairs*	No DNA synthesized		All DNA synthesized	
	$\frac{C_{2a}†}{C_{2b} \times 2}$	$\frac{C_{2b}}{C_{2a} \times 2}$	$\frac{C_{2a}}{C_{2b}}$	$\frac{C_{2b}}{C_{2a}}$
5	0.375–.399	0.667–.626	0.750–.798	1.333–1.252
8	.400–.424	.625–.589	.800–.848	1.250–1.178
21	.425–.499	.588–.556	.850–.898	1.176–1.112
20	.450–.474	.555–.527	.900–.948	1.110–1.054
29	.475–.499	.526–.501	.950–.998	1.052–1.002
83 Total				

* Of the 90 clones in Table II, 7 C_2 cells are represented by a single daughter; hence, only 83 pairs of cells are included in this table.

† In Table II, C_{2a} has been listed as the daughter with the lower value of DNA, and C_{2b} as the daughter with the higher value.

TABLE IV

Ratios of DNA in the 90 clones of Table II arranged in different time sequences

(A) Age of C_1 when fixed (min.)	$\frac{C_1\text{ DNA}}{C_2\text{ DNA}}$ ≤ 0.59	$\frac{C_1\text{ DNA}}{C_2\text{ DNA}}$ 0.60–.84	$\frac{C_1\text{ DNA}}{C_2\text{ DNA}}$ ≥ 0.85
		Number of clones	
30– 35	7	4	—
40– 65	3	7	1
70– 95	3	5	9
100–135	4	3	7
140–165	1	4	17
170–195	1	—	8
200–225	—	1	2
≥ 230	—	1	2
(B) Time to C_2 div. (after C_1 fixed) (min.)			
≥ 235	4	—	—
205–230	—	—	—
175–200	2	4	—
135–170	6	3	1
105–140	4	7	2
85–110	3	8	6
55– 80	—	3	13
25– 50	—	—	18
≤ 20	—	—	6
(C) $\frac{\text{Age of } C_1}{C_2 \text{ interphase}}$			
0.0–.09	1	—	—
.1–.19	5	2	—
.2–.29	5	6	—
.3–.39	1	4	2
.4–.49	4	3	1
.5–.59	3	4	5
.6–.69	—	3	10
.7–.79	—	3	10
.8–.89	—	—	12
.9–.99	—	—	6

very unequal division of DNA had occurred between the sister cells, thus masking the start of synthesis. In three other cases (Nos. 12, 15, 48), C_1 was fixed so early in interphase (65 minutes or less) that the low ratios probably have little significance. The last three clones (Nos. 82, 84, 85) are unexplained exceptions; possibly the apparent lack of synthesis in C_1 is related to the unusually long generation times.

The data obtained in the course of this study suggest that cells duplicate each unit of DNA during their growth cycles. The presence of 25 clones in the "partial synthesis" group (C_1/C_2 0.60 to 0.84) indicates that the synthesis is not instantaneous. In the "completed synthesis" group there are 43 clones with C_1/C_2 ratios of 0.85 to 1.17. (To be sure, some of the low ratios might represent C_1 cells still in the process of synthesis.) Of these 43 clones, 21 have ratios of 0.99 or less (indicating that C_1 contained the lower amount of DNA), 4 have ratios of 1.00, and 18 have ratios above 1.00 (indicating that C_1 contained the higher amount of DNA); selection of the cell with the lower or higher amount of DNA for C_1 was thus perfectly random. In 16 of these clones (37%) the sisters contained nearly equal amounts of DNA (C_1/C_2 ratios of 0.95–1.04); in 12 (28%) the ratios were less equal (0.90–0.94, and 1.05–1.10); and in 15 (35%) the ratios were even less equal (0.85–0.89, and 1.11–1.17). These figures take on special significance when compared with the 70 C_{2a}–C_{2b} cells falling in the same ratio groups (Table III, lines 3, 4, 5)—29 (41%) are in the first group, 20 (29%) in the second, and 21 (30%) in the third. Such close correspondence between these two sets of cells—sisters just at the end of the division process, and sisters of the ensuing life cycle—offers good evidence that, following division, the DNA content of a cell is precisely duplicated.

This investigation of DNA synthesis in *Tetrahymena* indicates, further, that a considerable time elapses from the completion of synthesis to the end of the interphase period. It is intriguing to consider the implications of this time period. Cytological examination shows no obvious, complicated mitotic apparatus being formed, although the chromatin does undergo a fairly regular condensation. Cellular reorganization, however, occurs before the mother cell divides into two daughters. The most striking change which takes place before the start of division is the formation of a new mouth (Furgason, 1940), allotted to the posterior daughter of the dividing pair. By studying growing cells under a phase microscope, it is hoped to determine the amount of time necessary for this process.

DISCUSSION

The synthesis of DNA by *Tetrahymena pyriformis* H clearly occurs during the interphase period. Other workers, in some cases using very different methods of analysis, have come to similar conclusions for cells with mitotically dividing nuclei—the micronuclei of a ciliate (*Chilodonella uncinatus*—Seshachar, 1950), vertebrate cells (Swift, 1950), chick fibroblasts in tissue culture (Walker and Yates, 1952), sea urchin embryos (McMaster, 1955), *Vicia faba* root tips (Howard and Pelc, 1951; Deeley *et al.*, 1957), onion root tips (Patau and Swift, 1953).

After the end of a division (the beginning of the new interphase) a period of time elapses before DNA synthesis begins. This pause was particularly obvious in some of the individual clones with long generation times, when several hours might elapse before synthesis could be detected. Synthesis appears to be completed more than an hour before the cells begin to divide, a considerable period when compared to their average generation time of around four hours.

The exact duplication of DNA during interphase is suggested strongly (1) in mass cultures, by the amounts of DNA in the macronuclei of dividing daughter cells during log phase, as compared with the amounts in older, non-dividing cells, and (2) in individual clones, by the similar DNA ratios in pairs of dividing daughters, as compared with those in sister pairs after synthesis of DNA had occurred.

In general, the amounts of DNA allotted to sister nuclei are remarkably close. Frequently, however, division is quite unequal; furthermore, very often a piece of chromatin is left behind in the cytoplasm. Such irregularities might be expected to result in genic imbalance, and death; instead, they seem to result in the wide range of DNA values found among non-clonal cells. Very occasional deaths have been noted among isolated cells (2 among a set of 101 cells, for example), which might be attributable either to cellular abnormality, or to some external factor. Among cells fixed during growth, occasional diffuse-looking nuclei have been found which were unsuitable for photometric analysis—possibly they represented dying cells, or possibly they resulted from faulty fixation. That unequal division of the macronucleus does not usually have an adverse effect is shown by the fact that the DNA ratios between apparently healthy sister cells, after synthesis of new DNA, are as variable as those between dividing daughters. It seems reasonable, however, that some method for a fairly orderly distribution of chromatin must be present in this micro-organism, with no micronucleus but only an amitotically dividing macronucleus, which has successfully propagated itself for almost 30 years in the laboratory. At division, the appearance of the *Tetrahymena* macronucleus does suggest some sort of organization.

Unfortunately, the basic structure of ciliate macronuclei is difficult to interpret. In *Paramecium aurelia,* however, Sonneborn (1947) obtained genetic evidence (the regeneration of parts of degenerating macronuclei) for genome segregation, from which he concluded that the macronucleus must contain about 40 diploid "subnuclei," distributed at amitotic division in intact units. (It will be recalled that Moses, 1950, estimated the macronucleus of *P. caudatum* to contain about 40 times as much nucleoprotein as the micronucleus.) Kimball (1953) could find no cytological evidence for subunits in the macronucleus of *P. aurelia,* however, but only for a high degree of polyploidy.

Grell, too, has been unable to find cytological evidence for subnuclei in the macronuclei of suctoreans (for example, Grell, 1953b). The budding by which these organisms reproduce vegetatively, however, suggests that genome segregation must occur. Particularly in the free-living stage of *Tachyblaston ephelotensis,* Grell (1950) noted the similarity in size and structure of the parts budded off the parent macronucleus, with its linear arrangement of chromatin (he reported that there appeared to be 8 chromatin elements in each bud). In another type of protozoan—a radiolarian, *Aulacantha scolymantha*—Grell (1953c) has found clear cytological evidence for a method by which genome segregation could occur. In the highly polyploid nucleus of this organism, the chromosomes appear to be linearly arranged in complete, individual sets, forming numerous chains of "Sammelchromosomen." Random separation of these intact genomes necessarily would result in perfectly balanced daughter nuclei. It is possible that the occurrence of "Sammel" chromosomes might also explain the efficiency of amitosis in ciliates, although the division figure of the *Aulacantha* nucleus, which also has no spindle, appears to be quite different from those of ciliate macronuclei.

Purely in the realm of speculation, it has occurred to the present author that, rather than "Sammel" chromosomes, the Feulgen-positive granular strands which appear to extend the length of the dividing macronucleus in *Tetrahymena* might each represent a row of identical chromosomes, held together by forces of attraction. If this were the case, the daughter nuclei would be assured of a fairly well balanced assortment of chromosomes, no matter how unequal the division (assuming that all the strands separated in approximately the same region). The chromatin fragments left behind at division would probably cause no serious imbalance, and might, indeed, be a way of correcting a nucleus somewhat imbalanced by the previous division. If a similar chromosome arrangement also occurred in the degenerating macronuclear skein of *Paramecium,* the parts breaking off would very likely contain complements of chromosomes, as has been suggested by the regeneration experiments. A condition such as this could also explain the efficiency of the budding of suctoreans.

Another characteristic of *T. pyriformis* H which has been demonstrated in the present experiments is the variability in generation times among different clones, as compared with the usual close similarity between sister cells. The length of generation time seems to have no relation to the amount of DNA.

Once synthesis of DNA has been completed, the photometric measurements of mass culture cells indicate that under some conditions division does not necessarily follow immediately. In general, cells from the one-week- and two-week-old culture contained approximately twice as much DNA as individual dividing daughters from log phase. Prescott's recent studies (1957) of generation time and lag phase in strain HS indicate that cells from stationary phase may divide soon after inoculation into fresh medium, and may then undergo a lag phase before logarithmic growth begins. Such preliminary division seems a reasonable consequence if the inoculated cells contained a doubled amount of DNA.

By alternating temperature changes, Scherbaum and Zeuthen (1954) caused logarithmically growing cells (strain GL) essentially to stop dividing until the final return to optimal temperature. The following synchronous (85%) division occurred 90 ± 10 minutes later, a time period remarkably similar to that found in the present experiments (about 80 minutes) to occur between the end of synthesis and the end of the interphase period. They report that the following two somewhat less synchronous divisions of the treated cells occurred about 1.7 hours (100 minutes) apart. Correlated with these interesting data is the fact that they found (Zeuthen and Scherbaum, 1954) by Hoff-Jørgensen microbiological assay that cells at the end of treatment contained about four times as much DNA as normally growing cells. By Schmitt-Thannhauser analysis, Ducoff (1956) has confirmed the unusual amount of DNA synthesis by temperature-treated cells. In view of the degree of DNA synthesis and the time elapsing before the first synchronous division, the question arises, as in the present experiments, about the length of time required for cellular changes (such as the formation of a new mouth) which must take place before division can begin.

The author wishes to express her appreciation to Professor A. W. Pollister, in whose laboratories this work was carried out, for his stimulating advice and guidance, and to Professor F. J. Ryan for the provision of additional laboratory facilities.

SUMMARY

1. In a mass culture of *Tetrahymena pyriformis* H which has stopped growing, the macronuclei contain approximately twice as much DNA as do newly divided macronuclei in a logarithmically growing culture.

2. Non-clonal cells show considerable variability as to DNA content and generation time.

3. Cells in small clones show close similarity as to DNA content and generation time.

4. Duplication of DNA occurs during an intermediate part of interphase, starting some time after the end of the previous cell division, and reaching completion a considerable period of time before the next division begins.

LITERATURE CITED

Alfert, M., and I. I. Geschwind, 1953. A selective staining method for the basic proteins of cell nuclei. *Proc. Nat. Acad. Sci.*, **39**: 991–999.

Alfert, M., and N. O. Goldstein, 1955. Cytochemical properties of nucleoproteins in *Tetrahymena pyriformis;* a difference in protein composition between macro and micronuclei. *J. Exp. Zool.*, **130**: 403–421.

Chen, T. T., 1940. Conjugation in *Paramecium bursaria* between animals with very different chromosome numbers and between animals with and without micronuclei. *Proc. Nat. Acad. Sci.*, **26**: 243–246.

Chen, T. T., 1944. Staining nuclei and chromosomes in protozoa. *Stain Tech.*, **19**: 83–90.

Corliss, J. O., 1953. Comparative studies on holotrichous ciliates in the *Colpidium-Glaucoma-Leucophrys-Tetrahymena* group. II. Morphology, life cycles and systematic status of strains in pure culture. *Parasitology*, **43**: 49–87.

Deeley, E. M., H. G. Davies and J. Chayen, 1957. The DNA content of cells in the root of *Vicia faba*. *Exp. Cell Res.*, **12**: 582–591.

Di Stefano, H. S., 1948. A cytochemical study of the Feulgen nucleal reaction. *Chromosoma*, **3**: 282–301.

Ducoff, H. S., 1956. Radiation-induced fission block in synchronous cultures of *Tetrahymena pyriformis* W. *Exp. Cell Res.*, **11**: 218–220.

Elliott, A. M., and R. E. Hayes, 1953. Mating types in *Tetrahymena*. *Biol. Bull.*, **105**: 269–284.

Furgason, W., 1940. Significant cytostomal pattern of "*Glaucoma-Colpidium* group" and a proposed new genus and species, *Tetrahymena geleii*. *Arch. f. Protist.*, **94**: 224–266.

Grell, K. G., 1950. Der Generationswechsel des parasitischen Suktors *Tachyblaston ephelotensis* Martin. *Zeitschr. f. Parasit.*, **14**: 499–534.

Grell, K. G., 1953a. Die Konjugation von *Ephelota gemmipara* R. Hertwig. *Arch. f. Protist.*, **98**: 287–326.

Grell, K. G., 1953b. Die Struktur des Makronucleus von *Tokophrya*. *Arch. f. Protist.*, **98**: 466–468.

Grell, K. G., 1953c. Die Chromosomen von *Aulacantha scolymantha* Haeckel. *Arch. f. Protist.*, **99**: 1–54.

Hetherington, A., 1933. The culture of some holotrichous ciliates. *Arch. f. Protist.*, **80**: 255–280.

Howard, A., and S. R. Pelc, 1951. Nuclear incorporation of P^{32} as demonstrated by autoradiographs. *Exp. Cell Res.*, **2**: 178–187.

Kidder, G. W., V. C. Dewey and M. R. Heinrich, 1954. The effect of non-ionic detergents on the growth of *Tetrahymena*. *Exp. Cell Res.*, **7**: 256–264.

Kimball, R. F., 1953. The structure of the macronucleus of *Paramecium aurelia*. *Proc. Nat. Acad. Sci.*, **39**: 345–347.

McMaster, R. D., 1955. Desoxyribose nucleic acid in cleavage and larval stages of the sea urchin. *J. Exp. Zool.*, **130**: 1–27.

Moses, M. J., 1950. Nucleic acids and proteins in the nuclei of *Paramecium*. *J. Morph.*, **87**: 493–536.

Nanney, D. L., 1953a. Mating type determination in *Paramecium aurelia*, a model of nucleocytoplasmic interaction. *Proc. Nat. Acad. Sci.*, **39**: 113–119.

Nanney, D. L., 1953b. Nucleocytoplasmic interaction during conjugation in *Tetrahymena*. *Biol. Bull.*, **105**: 133–148.

Nanney, D. L., 1956. Caryonidal inheritance and nuclear differentiation. *Amer. Nat.,* **90**: 291–307.

Nanney, D. L., 1957. The role of cytoplasm in heredity. *In:* A Symposium on the Chemical Basis of Heredity, W. D. McElroy and B. Glass, eds., The Johns Hopkins Press, Baltimore, 134–164.

Nanney, D. L., and P. A. Caughey, 1953. Mating type determination in *Tetrahymena pyriformis. Proc. Nat. Acad. Sci.,* **39**: 1057–1063.

Ornstein, L., 1952. The distributional error in microspectrophotometry. *Lab. Invest.,* **1**: 250–265.

Patau, K., 1952. Absorption microphotometry of irregular-shaped objects. *Chromosoma,* **5**: 341–362.

Patau, K., and H. Swift, 1953. The DNA content (Feulgen) of nuclei during mitosis in a root tip of an onion. *Chromosoma,* **6**: 149–169.

Pollister, A. W., 1952. Photomultiplier apparatus for microspectrophotometry of cells. *Lab. Invest.,* **1**: 106–114.

Prescott, D. M., 1957. Change in physiological state of cell population as a function of culture growth and age (*Tetrahymena geleii*). *Exp. Cell Res.,* **12**: 126–134.

Scherbaum, O., and E. Zeuthen, 1954. Induction of synchronous cell division in mass cultures of *Tetrahymena pyriformis. Exp. Cell Res.,* **6**: 222–227.

Seshachar, B. R., 1950. Desoxyribonucleic acid content of the ciliate micronucleus. *Nature,* **165**: 848.

Seshachar, B. R., and C. M. S. Dass, 1953. Macronuclear regeneration in *Epistylis articulata. Quart. J. Micr. Sci.,* **94**: 185–192.

Sonneborn, T. M., 1939. *Paramecium aurelia:* mating types and groups; lethal interactions; determination and inheritance. *Amer. Nat.,* **73**: 390–413.

Sonneborn, T. M., 1947. Recent advances in the genetics of *Paramecium* and *Euplotes. Adv. in Genetics,* **1**: 263–358.

Sonneborn, T. M., 1951. Some current problems of genetics in the light of investigations on *Chlamydomonas* and *Paramecium. Cold Spring Harb. Symp. Quant. Biol.,* **16**: 483–503.

Sonneborn, T. M., 1953. Patterns of nucleocytoplasmic integration in *Paramecium. Proc. 9 Intern. Congr. Genetics, Caryologia,* **6** (Suppl. I): 307–325.

Sonneborn, T. M., 1954. Is gene K active in the micronucleus of *Paramecium? Microb. Genet. Bull.,* **11**: 25–26.

Swift, H., 1950. The desoxyribose nucleic acid content of animal nuclei. *Physiol. Zoöl.,* **23**: 169–198.

Swift, H., 1953. Quantitative aspects of nuclear nucleoproteins. *Int. Rev. Cyt.,* **2**: 1–76.

Walker, P. M. B., and H. B. Yates, 1952. Nuclear components of dividing cells. *Proc. Roy. Soc. London, Ser. B,* **140**: 274–299.

Wichterman, R., 1953. The Biology of Paramecium. The Blakiston Co., New York.

Zeuthen, E., and O. Scherbaum, 1954. Synchronous divisions in mass cultures of the ciliate protozoan *Tetrahymena pyriformis* as induced by temperature changes. *In:* Recent Developments in Cell Physiology, J. A. Kitching, ed.; Proceedings of the 7th Symposium of the Colston Research Society, London, 141–157.

CONTRACTILE PROTEIN FROM CRAYFISH TAIL MUSCLE

K. MARUYAMA

Biological Institute, College of General Education, University of Tokyo,
Komaba, Meguro, Tokyo, Japan

Physicochemical studies on vertebrate striated muscle clearly indicate that the interaction of actomyosin, the muscle contractile protein, with adenosine triphosphate (ATP) may be the fundamental phenomenon on the molecular level in muscular contraction (*cf.* Szent-Györgyi, 1951; Weber and Portzehl, 1954). More and more evidence to support this thesis has been obtained in the field of comparative biochemistry in the animal kingdom (Maruyama and Tonomura, 1957).

In invertebrates, the highly developed muscles of arthropods and molluscs have been investigated biochemically in detail. In arthropods, however, there are very few reports on crustacean contractile protein, whereas the characteristics of insect actomyosin have been well established (Gilmour and Calaby, 1953; Maruyama, 1954, 1957b, 1957c). Edsall and Mehl (1940) investigated flow birefringence properties of lobster myosin in relation to the protein denaturation. Humphrey (1948) prepared myosin from the crab, *Maia,* and briefly described its adenosinetriphosphatase (ATPase) properties. Shrinkage of the glycerine-treated muscle fibers of *Limulus* with ATP was observed by Sarkar (1950). On the physicochemical properties of crustacean tropomyosin, detailed works have been recently published (Tsao, Tan and Peng, 1956; Laki, 1957; Kominz, Saad and Laki, 1957).

The present article is concerned with the results of a comparative biochemical study on the ATP-myosin B system and several associated enzymes in crayfish tail muscle.

MATERIALS AND METHODS

Materials. The crayfish, *Cambarus clarkii,* was used as material. Tail muscles were carefully isolated from exoskeleton and well washed with cold de-ionized water. In a few cases, jaw muscles were also dissected out.

Preparation of contractile protein. The so-called myosin B or natural actomyosin was extracted and purified as established in rabbit skeletal muscle (Szent-Györgyi, 1945). Muscles were homogenized in ten times the volume of cold 0.05 M KCl in a Waring Blendor and the water-extractable portion was removed by centrifugating at 3000 G for 5 minutes at 0° C. The residue was washed twice more and finally suspended in five times the volume of the Weber-Edsall solution (0.6 M KCl, 0.04 M $NaHCO_3$, 0.01 M Na_2CO_3). After being placed for 24 hours at 0° C., the suspension turned so viscous that it was diluted with an equal volume of 0.6 M KCl. The suspension was centrifuged for 10 minutes to remove the insoluble matter. The viscous supernatant was neutralized to pH 6.5–6.8 with dilute acetic acid and diluted in ten times the volume of cold de-ionized water. The flocculant precipitate was collected by centrifugation, dissolved in 0.6 M KCl and diluted in water. This dilution-precipitation procedure was repeated three times and finally the substances insoluble in 0.6 *M* KCl were removed by centrifugation at 8000 G for 30 minutes at 0° C.

Methods of assaying physicochemical properties. The measurements of ultraviolet absorption spectra were carried out with a Shimazu spectrophotometer. Purine and pentose contents were estimated according to Schneider's procedure (Schneider, 1946). Solubility in KCl was tested at pH 7.0 at 0° C., as described in an earlier paper (Maruyama, 1957a). Salting-out analysis was carried out according to Snellman and Tenow (1954), as modified by Tonomura and his associates (1956).

Methods of observing physical changes with ATP. Super-precipitation was observed in a test tube, containing 0.03 *M* tris-(hydroxymethyl)-aminomethane (Tris) buffer, 1 mM ATP and 1.5–2.5 mg. protein at pH 7.0 and 25° C. Viscosity was measured with viscosimeters of the usual Ostwald type at pH 6.4 at 5–10° C. Turbidity was determined in a Hitachi turbidimeter at pH 6.4 and 15° C. For investigation of viscosity and turbidity, the concentration of myosin B was suitably adjusted by dilution with 0.6 *M* KCl. A small amount of concentrated ATP was added to test the ATP effect.

Enzyme tests. The ATPase activity was determined by measuring the increase in inorganic phosphorus (P) after a specified time, usually 5 minutes at 30° C., in a system containing the protein dissolved in 0.6 *M* KCl, 1 mM ATP, 0.033 *M* Tris buffer (pH 7.0), 0.6 *M* KCl, and 10 mM $CaCl_2$ or some other ions, as specified. Total volume of the reaction mixture was 1.5 ml. In order to investigate the effect of pH of incubation, 0.05 *M* histidine was substituted for Tris. The reaction was started by the addition of substrate and stopped by the addition of 0.5 ml. of 20% trichloroacetic acid (TCA). Appropriate blanks were always run simultaneously with the experimentals.

The water-extractable apyrase activity was determined on the 0.05 *M* KCl extract of muscle suspensions. On proving the occurrence of adenylate kinase in crayfish muscle, the 0.05 *M* KCl extract was treated with heat and acid according to Colowick and Kalckar (1943) and incubated in the ATPase-assaying system with and without crayfish myosin B. Adenylate deaminase activity was tested as described before (Maruyama, 1957a).

ATP was purchased as crystalline disodium salt from Sigma and AMP from Schwarz Labs.

Protein was estimated by multiplying nitrogen values, obtained by a micro-Kjeldahl procedure, by the factor 6.

Inorganic phosphorus was measured on a 1.0-ml. aliquot of the TCA supernatant by a micro-modification of the method of Lohmann and Jendrassik (1926), as described by Moriwaki (1956).

RESULTS

Some Physicochemical Properties

Absorption spectra. Ultraviolet absorption spectra of crayfish myosin B dissolved in 0.6 *M* KCl showed the characteristics of protein nature: a maximal absorption was found around 275 mμ and a minimal one was at 255 mμ. Extinction coefficient (ε) on basis of gram N per *l*, and 1.0 cm. light path was 9.0 at 275 mμ, which was somewhat higher than that of rabbit myosin B (Tarver and Morales, 1951). The ratio of absorption coefficient at 275 mμ to that at 255 mμ is 1.3.

Purine and pentose contents. The results described above suggest that some minute amounts of purine-containing substances such as nucleotides or nucleic acids were present as contaminants in the preparations. The acid-soluble nucleotide fraction contained 1.5×10^{-5} moles purine per gram myosin B and the nucleic acid fraction contained 1.0×10^{-5} moles. The determinations of pentose showed approximately equimolar amounts and any detectable amounts of desoxyribose were not present. These values are a little higher than those for rabbit myosin B (Buchthal *et al.*, 1951).

Solubility in KCl. Crayfish myosin B was completely soluble in concentrations of KCl higher than 0.4 *M*. The solubility curve is quite in accord with that of rabbit myosin B (Szent-Györgyi, 1945). Myosin B dissolved in 0.6 *M* KCl was slightly yellowish white in color.

TABLE I

Effect of varied concentrations of Ca, Mg and EDTA on the super-precipitation of crayfish myosin B

Concn. *M*	Ca	Mg	EDTA
0	++	++	++
10^{-6}	++	++	++
10^{-5}	++	+++	+
10^{-4}	++	±	±
10^{-3}	+	−	−
10^{-2}	±	−	−

Observed within three minutes after the addition of 1 mM ATP at pH 7.0 and 20° C., in the presence of 0.10 *M* KCl.

+++, ++: intense; +: moderate; ±: weak; −: negative.

Salting-out analysis. Under experimental conditions similar to those of Snellman and Tenow (1954), nearly all the proteins were precipitated between 30–38% of saturated ammonium sulfate in the present preparation.

Physical Changes with ATP

Super-precipitation. Under the optimal conditions, *e.g.*, at 20° C. and pH 7.0 in the presence of 0.10 *M* KCl, a typical super-precipitation was found to take place within one minute after the addition of 1 mM ATP, and to reach the end within five minutes. The super-precipitation was evidently recognized in the presence of KCl in concentrations between 0.06 and 0.16 *M*. The optimal KCl concentrations were 0.10–0.12 *M*, which were quite in accordance with those of rabbit myosin B (Szent-Györgyi, 1945). The effects of Ca, Mg and ethylenediaminetetraacetic acid (EDTA), were tested (Table I). These agents in a high concentration (10 mM) inhibited the super-precipitation. Calcium ions affected little, having no accelerating effect. Magnesium ions greatly speeded up the precipitation in 10^{-5} *M*, but retarded in over 10^{-4} *M*. On the other hand, EDTA inhibited in concentrations higher than 10^{-4} *M*. It should be noted that the inhibitory effects of high concentrations of Mg or Ca and of EDTA were qualitatively different: after several hours, the precipitation took place even in the presence of a high concentration of Mg or Ca, but in the presence of EDTA no precipitation was observed to occur.

Change of viscosity. The relative viscosity of crayfish myosin B dissolved in 0.6 M KCl was highly anomalous and ATP caused a marked drop in the viscosity (Fig. 1).

According to Weber and Portzehl (1952), the extent of the viscosity change of actomyosin with ATP (ATP sensitivity) can be expressed as follows:

$$\text{ATP sensitivity} = \frac{Z\eta - Z\eta_{\text{ATP}}}{Z\eta_{\text{ATP}}} \times 100$$

Here $Z\eta = \ln\eta\text{rel}/C$; ηrel = relative viscosity in the absence of ATP; $Z\eta_{\text{ATP}}$ = that in the presence of a sufficient amount of ATP to cause the maximal viscosity change; and C = concentration of protein (grams/liter). The viscosity data derived from Figure 1 are as follows: $Z\eta = 0.40$, $Z\eta_{\text{ATP}} = 0.20$ and the ATP sensitivity = 100%.

The viscosity of myosin B solution drops rapidly with the addition of ATP and gradually rises again when ATP is split by the ATPase action of myosin B. The recovery process followed an S-shaped curve and the recovered viscosity reached about 50% of the drop. Calcium ions, which activate the ATPase action, strongly accelerated the recovery process, while magnesium, an inhibitor of the ATPase action, retarded it. These tendencies are in good accord with those in rabbit or insect myosin B (*cf.* Mommaerts, 1948; Maruyama, 1957b). On the other hand, EDTA, in 10 mM, completely inhibited the viscosity change with ATP.

Change of turbidity. On addition of ATP, the apparent turbidity of myosin B solution decreases because of the decrease of intensity of scattered light (*cf.* Tonomura, 1956). Figure 2 shows the change of turbidity in crayfish myosin B, which

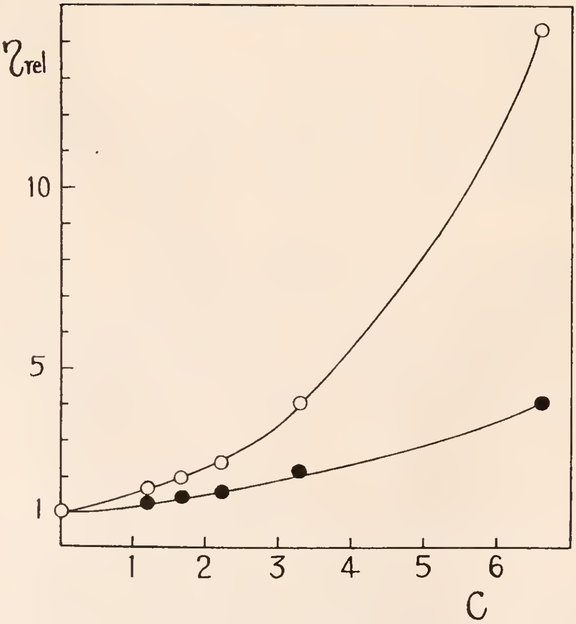


FIGURE 1. Drop of viscosity of crayfish myosin B with ATP; pH 6.4; 6° C.; 0.6 M KCl. ○, control; ●, 1 mM ATP added. C, protein concentration, g./l.

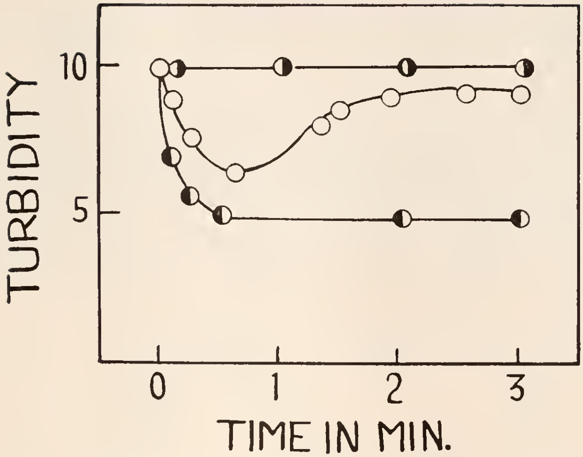


FIGURE 2. Change of turbidity of crayfish myosin B with ATP; pH 6.4; 15° C.; 1 mM ATP; 0.6 *M* KCl. ◑, 10 mM EDTA; ○, 10 mM $CaCl_2$; ◐, 10 mM $MgCl_2$. Turbidity is given as relative level. Time, after the addition of ATP.

is qualitatively similar to that of viscosity described above. The maximal drop of turbidity reached 50% of the original level.

Enzyme Activity

Water-extractable Apyrase

Muscle contains at least two kinds of ATP-splitting enzymes, one myosin itself and the other the water-extractable, magnesium-activated enzyme(s). In crayfish muscle, there was found the latter enzyme(s), too. This fact was already suggested by the classical work of Lohmann (1935) on lobster muscle. The apyrase activity was greatly activated by Mg and completely inhibited by Ca ions. For example, the water-extract, containing 0.3 mg. protein, liberated the following amount of P (μg.) from 2.4 μmole ATP in five minutes at pH 7.0 and 30° C.: 7.0 (none added); 22.0 (5 mM $MgCl_2$); 3.0 (5 mM $CaCl_2$); 2.5 (5 mM $MgCl_2$ plus 5 mM $CaCl_2$). The water-extract of crayfish muscle easily hydrolyzed the two phosphate groups from ATP in the presence of Mg ions.

Adenylate Kinase

In the water-extract of crayfish muscle, adenylate kinase was found to exist. The heat- and acid-treated sample showed no ATPase activity, but in the presence of myosin B, ATPase hydrolyzed the two phosphate groups from ATP. In the presence of 10 mM EDTA, only the terminal phosphate group was set free, even in the combined action of myosin B and the extract. This may be due to the inhibition of adenylate kinase by EDTA, as is well known in rabbit or rat muscle (*cf.* Bowen and Kerwin, 1954).

Adenylate Deaminase

No 5-adenylic acid deaminase activity was detected in crayfish myosin B under the present experimental conditions. This absence of adenylate deaminase activity in crayfish myosin B coincides with the results of Lohmann (1935) on lobster muscle homogenates.

ATPase

Crayfish myosin B possessed a typical calcium-activated ATPase activity, as well as myosin B's from other animal muscles. Preliminary experiments showed that the ATPase action at pH 7.0 was optimal around 30–35° C. and at 37° C. the enzyme was soon inactivated.

Effect of pH. The effect of pH of incubation on the ATPase action of crayfish myosin B is indicated in Figure 3. In the presence of 10 mM $CaCl_2$ in addition to 0.62 *M* KCl and 0.05 *M* histidine, two pH optima were evident: a higher one, at pH 9.0 and a lower one at pH 6.0. In the presence of 10 mM EDTA, a striking activator, a flat, but very high pH optimum between 7–8.5 was found (Fig. 3). The activity level, expressed in terms of Qp (Engelhardt, 1946) is approximately as follows: 1300 at pH 6.0, 600 at pH 7.0 and 2000 at pH 9.0 in the presence of 10 mM $CaCl_2$ and 0.6 *M* KCl at 30° C.; more than 3000 at pH 7–8 in the presence of 10 mM EDTA and 0.6 *M* KCl at 30° C. These values are of a similar magnitude to those reported in actomyosin ATPase from other animals (*cf.* Maruyama and Tonomura, 1957).

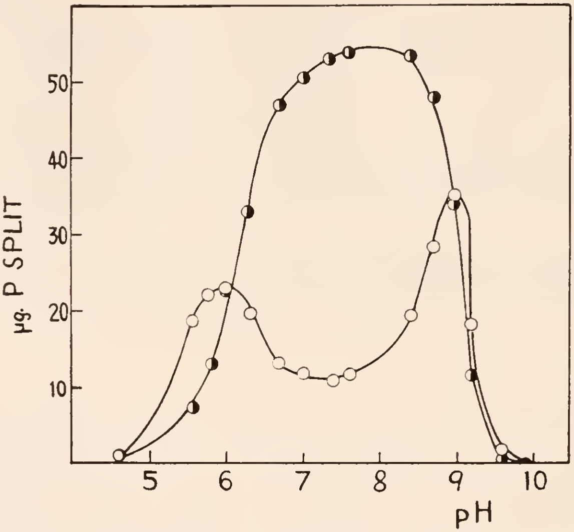


FIGURE 3. Curve of pH-activity of crayfish myosin B ATPase; 0.05 *M* histidine buffer, 30° C.; 5 min. incubation; 0.62 *M* KCl; 2.4 μM ATP; 0.16 mg. protein. ○, 10 mM $CaCl_2$; ◑, 10 mM EDTA.

Effect of enzyme concentration and incubation time. The enzyme activity was found to be linear to enzyme concentration and incubation time, when the hydrolysis of the substrate surpassed no more than half of the added amount. As is seen in Figure 4, it is apparent that in the presence of 10 mM Ca or EDTA, no phosphate was set free after one-half of the labile phosphates corresponding to the terminal phosphate level of ATP were hydrolyzed. In the presence of Mg ions, the activity proceeded at a slight, but constant, rate up to 60 minutes. It is clear that crayfish myosin B exhibits a true ATPase action as well as established in the rabbit.

Effect of Ca, Mg and EDTA. The effects of K, Mg, Ca and EDTA on the ATPase activity of crayfish myosin B were investigated in a systematic way. In dilute KCl concentrations of KCl, *e.g.,* 0.1 *M,* the enzyme action was found to be irregular, possibly owing to the remarkable super-precipitation of the protein. However, it is at least sure that in the presence of 0.1 *M* KCl, $MgCl_2$ at 10^{-5} *M* inhibited the ATPase action, at 10^{-3} *M* it elevated the enzyme activity to the original level and at 10^{-2} *M* it again decreased the activity. In the presence of 0.6 *M* KCl, magnesium depressed the ATPase activity increasingly as the concentration became higher from 10^{-5} to 10^{-2} *M*. On the other hand 0.01–0.1 *M* $CaCl_2$ activated ten times the enzyme action, irrespective of the KCl concentration. Mg competitively inhibited the activating effect of Ca.

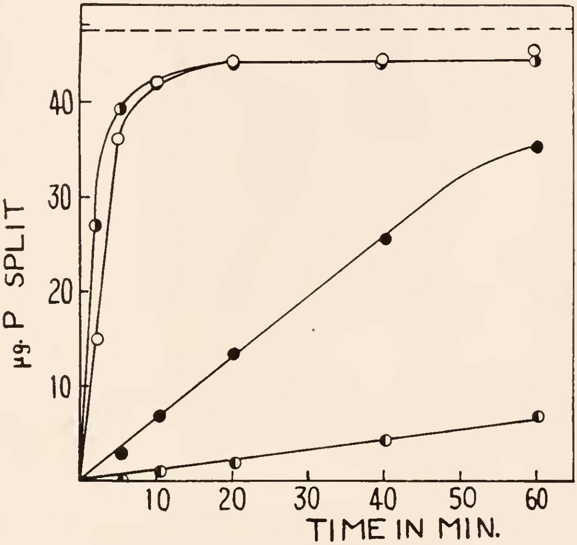


FIGURE 4. Time-activity curve of crayfish myosin B ATPase; pH 7.0; 30° C.; 0.52 *M* KCl; ATP = 48 μg. 1/2 △7P (level of dotted line); 0.5 mg. protein. ●, none added; ◐, 10 mM $MgCl_2$; ○, 10 mM $CaCl_2$; ◑, 10 mM EDTA.

The accelerating effect of EDTA was most remarkable in the presence of 0.6 *M* KCl; 0.6 *M* NaCl could not substitute for KCl. EDTA was inactive in 0.1 mM and gave the strong activation in concentrations higher than 0.5 mM and optimal in 10 mM (see Fig. 5). The effects of Ca, Mg and EDTA on the ATPase activity are seen in Figure 4.

Effect of substrate concentration. The effect of increasing substrate concentration is summarized in Figure 5. The enzyme reaction proceeded according to the Michaelis-Menten theory:

$$\frac{1}{v} = \frac{1}{Vm} + \frac{Km}{Vm}\frac{1}{S}$$

Here v = reaction velocity of first order, Vm = maximal reaction rate, Km = Michaelis constant and S = substrate concentration, Michaelis constant (Km) was approximately 5×10^{-5} *M* (in the presence of 10 mM Ca or 0.1 mM EDTA), 3×10^{-4} *M* (1 mM EDTA) and 1×10^{-3} *M* (10 mM EDTA). Maximal velocity (Vm) also became much greater in the presence of EDTA, as the concentration increased. In the presence of 10 mM Ca ions, Km changed little, although Vm appreciably increased. These facts are in good harmony with those in the rabbit (Tonomura, Matsumiya and Kitagawa, 1957).

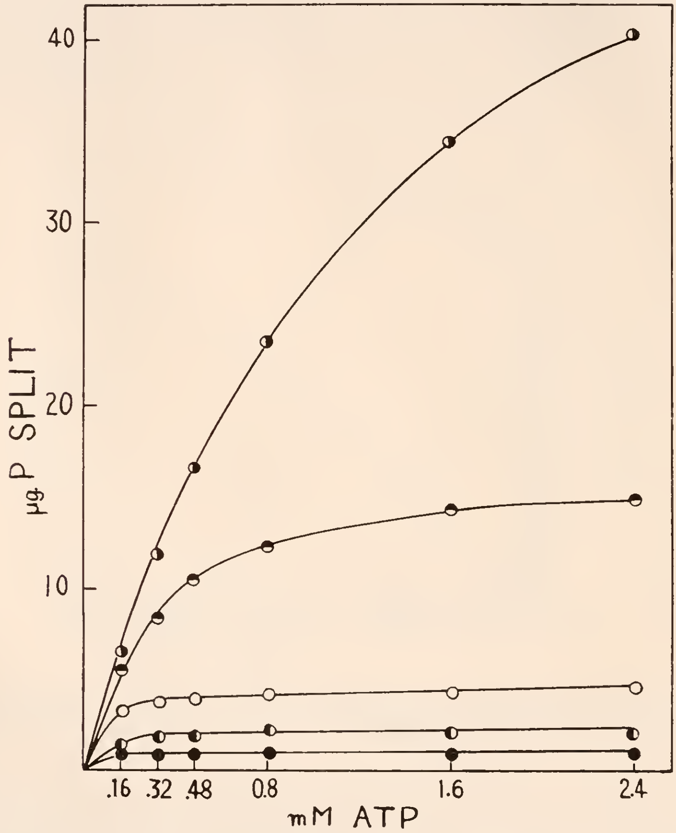


FIGURE 5. Effect of substrate concentration on the ATPase activity of crayfish myosin B; pH 7.0; 30° C.; 3 min. incubation; 0.52 *M* KCl; 0.16 mg. protein. ●, none added; ○, 10 mM $CaCl_2$; ◐, 0.1 mM EDTA; ◓, 1 mM EDTA; ◑, 10 mM EDTA.

Effect of SH reagents. The ATPase activity of crayfish myosin B was very sensitive to heavy metals such as zinc or copper. Especially $ZnSO_4$ in 1 mM completely inactivated the enzyme action. The poisoning action of heavy metals was thought to be mediated through SH groups of myosin B, so that the effect of P-chloromercuribenzoate (PCMB), the powerful SH-blocking agent, was tested; 10^{-4} *M* PCMB nullified the ATPase activity without any pretreatment. After myosin

B was deactivated on a few minutes' pretreatment with 10^{-4} M PCMB, glutathione (GSH) was added and after 5 minutes at 20° C., the ATPase action was started by the addition of ATP. GSH in 10^{-3} M recovered 10% of the original activity and in 10^{-2} M 35% of that was restored. On the other hand, PCMB in a dilute concentration of $5\text{–}10 \times 10^{-6}$ M rather enhanced the ATPase activity by some 10–30% in the presence of 10 mM Ca as activator. The latter fact confirms the recent finding of Kielley and Bradley (1956) on rabbit myosin ATPase. These influences of PCMB and GSH show that SH groups may be involved in the active center of the ATPase action in the protein molecule.

Discussion

From the observations described above, the interaction of myosin B from crayfish tail muscle with ATP is quite the same as that of rabbit myosin B. The great extent of physical changes, e.g., super-precipitation, drop of viscosity or of turbidity with ATP, shows the main component of the present myosin B preparation was actomyosin. The salting-out analysis suggested that most of the proteins might be actomyosin and a minor part of them be myosin. There is no evidence to show the presence of tropomyosins as contaminants in the preparation. The thread prepared from crayfish myosin B contracted on addition of ATP. It is to be noted that myosin B from crayfish jaw muscle was very similar to that from tail muscle with respect to the ATPase activity, and super-precipitation and viscosity change with ATP.

Attention should be arrested to the pH optima of the ATPase activity of crayfish myosin B. The pH-activity curve in the presence of 10mM $CaCl_2$ and 0.6 M KCl is quite similar to that of molluscan myosin B (Maruyama, 1957a), not to that of insects (Maruyama, 1954). This is rather of the vertebrate type (*cf.* Engelhardt, 1946). In any case, two pH optima in the presence of Ca, activation by Ca and inhibition of the Ca effect by Mg in the ATPase action are all common to myosin B's from higher invertebrate and all vertebrate muscles so far reported (*cf.* Maruyama and Tonomura, 1957), although the former seems not to be the case in myosin ATPase from squid mantle muscle, where only the acid optimum was observed (de Villafranca, 1955).

Although essentially the same regards with the interaction between the contractile protein and ATP, one definite difference exists between actomyosins from invertebrate and vertebrate muscles. This is the absence of adenylate deaminase activity in invertebrate actomyosins. This fact was first discovered in insect muscle by Gilmour and Calaby (1953) and later confirmed in insects (Maruyama, 1957c), a sea-anemone (Maruyama, 1957d) and molluscs (Kitagawa and Tonomura, 1957; Maruyama, 1957a). These comparative studies may throw a strong doubt on the essential physiological role of adenylate deaminase in muscular activity. From the viewpoint of biochemical evolution, it is of much interest to investigate whether adenylate deaminase activity is present in prochordate muscles, especially in Acrania.

The relatively large quantity of easily available muscle of crayfish tail will make it a highly desirable material for a further study of the biochemistry and biophysics of the muscle contractile proteins from the comparative point of view. Crayfish myosin B was shown to possess the most similar peculiarities to rabbit in invertebrates so far known (*cf.* Maruyama and Tonomura). Especially the role of the guanidine kinase system in the recovery process of the ATP-induced physical change of the actomyosin solution, suggested by Tonomura's school (1956) in *Pecten* adductors, is expected to be elucidated more distinctly in crayfish tail muscle.

SUMMARY

1. Contractile protein (myosin B) was extracted and purified from tail muscle of the crayfish *Cambarus clarkii.*

2. Ultraviolet absorption spectra of crayfish myosin B dissolved in 0.6 *M* KCl showed a protein nature. The $\epsilon 275$ was 9 and $\epsilon 275/\epsilon 255$ was 1.3. Acid-soluble purine-pentose content was 1.5×10^{-5} mole per gram protein and nucleic acid purine-pentose content was 1×10^{-5} mole.

3. Crayfish myosin B was quite soluble in 0.4 *M* KCl. Salting-out analysis with ammonium sulfate indicated the main component is actomyosin.

4. Super-precipitation with ATP was clearly seen to take place in the range of 0.08–0.16 *M* KCl concentrations. The phenomenon was most distinguished in the presence of 0.1 *M* KCl and 10^{-5} *M* $MgCl_2$ at pH 7.0 at 25° C. EDTA, in concentrations higher than 10^{-4} *M*, completely inhibited the precipitation.

5. A drop of viscosity with ATP was observed in the presence of 0.6 *M* KCl. The ATP sensitivity of Weber and Portzehl was 100%. The recovery process was also observed to a considerable extent.

6. ATP also caused a drop of turbidity of the myosin B solution, the maximal drop reaching 50%.

7. The magnesium-activated apyrase activity and adenylate kinase activity were detected in the water-extract of crayfish muscle.

8. No adenylate deaminase activity was demonstrated in crayfish myosin B.

9. Crayfish myosin B had a true ATPase, activated by Ca. In the presence of 0.6 *M* KCl and 10 mM Ca at 30° C., the enzyme showed two pH optima, a higher one at 9.0 and a lower one at 6.0. In the presence of 0.6 *M* KCl at pH 7–8, EDTA maximally enhanced the ATPase action, Qp being higher than 3000. The activating effect of Ca was sensitive to the inhibiting action of Mg. The Michaelis constant was 5×10^{-5} *M* in the presence of 10 mM Ca and 1×10^{-3} *M* in the presence of 10 mM EDTA.

10. The ATPase action of crayfish myosin B was very sensitive to heavy metals, such as Cu or Zn. PCMB easily blocked the enzyme action. However, PCMB in dilute concentrations rather enhanced the Ca-activated ATPase activity.

LITERATURE CITED

BOWEN, W. J., AND T. D. KERWIN, 1954. A study of the effects of ethylenediaminetetraacetic acid on myosin adenosinetriphosphatase. *J. Biol. Chem.*, **291**: 237–247.

BUCHTHAL, F., A. DEUTSCH, G. G. KNAPPEIS AND A. MUNCH-PETERSEN, 1951. Further investigations on the phosphorus and nucleotide uptake of actomyosin. *Acta Physiol. Scand.*, **24**: 365–384.

COLOWICK, S. P., AND H. M. KALCKAR, 1943. The role of myokinase in transphosphorylations. 1. The enzymatic phosphorylation of hexoses by adenylpyrophosphate. *J. Biol. Chem.*, **148**: 117–126.

EDSALL, J. T., AND J. W. MEHL, 1940. The effect of denaturing agents on myosin. II. Viscosity and double refraction of flow. *J. Biol. Chem.*, **133**: 409–429.

ENGELHARDT, V. A., 1946. Adenosinetriphosphatase properties of myosin. *Adv. in Enzymol.*, **6**: 147–192.

Gilmour, D., and J. H. Calaby, 1953. Physical and enzymatic properties of actomyosins from the femoral and thoracic muscles of an insect. *Enzymologia,* **16**: 23–33.

Humphrey, G. F., 1948. The adenosinetriphosphatase activity of myosins from marine animals. *Physiol. Comp. et Oecol.,* **1**: 89–94.

Kielley, W. W., and L. B. Bradley, 1956. The relationship between sulfydryl groups and the activation of myosin ATPase. *J. Biol. Chem.,* **218**: 653–659.

Kitagawa, S., and Y. Tonomura, 1957. Contractile proteins from adductors of *Pecten.* III. Failure to detect adenylic acid deaminase activity. *J. Biochem.* (*Tokyo*), **44**: 317–320.

Kominz, D. R., F. Saad and K. Laki, 1957. Vertebrate and invertebrate tropomyosins. *Nature,* **179**: 206–207.

Laki, K., 1957. Composition of contractile muscle proteins *J. Cell. Comp. Physiol.,* (in press).

Lohmann, K., 1935. Über die Aufspalung der Adenylpyrophosphorsäure und Argininphosphorsäure in Krebsmuskulatur. *Biochem. Zeitschr.,* **282**: 109–119.

Lohmann, K., and L. Jendrassik, 1926. Kolorimetrische Phosphorsäure-Bestimmungen im Muskelextrakt. *Biochem. Zeitschr.,* **178**: 419–426.

Maruyama, K., 1954. Studies on adenosinetriphosphatase from various insect muscles. *J. Fac. Sci. Tokyo Univ.,* IV, **7**: 231–270.

Maruyama, K., 1957a. Contractile proteins from adductors of *Meretrix* and *Cristaria. Annot. Zool. Jap.,* **30**: 63–66.

Maruyama, K., 1957b. Interaction of insect actomyosin with adenosine triphosphate. *J. Cell. Comp. Physiol.,* (in press).

Maruyama, K., 1957c. A further study of insect actomyosin. *Sci. Pap. Coll. Gen. Educ. Univ. Tokyo,* **7**: 213–241.

Maruyama, K., 1957d. Sea-anemone apyrase. I. Identification of the reaction products. *Enzymologia,* (in press).

Maruyama, K., and Y. Tonomura, 1957. Comparative studies of myosin B-ATP system. *J. Res. Inst. for Catalysis* (*Hokkaido Univ.*), **5**: 55–70.

Mommaerts, W. F. H. M., 1948. The reaction between actomyosin and adenosine triphosphate. *J. Gen. Physiol.,* **31**: 361–375.

Moriwaki, K., 1956. Effects of azide on the ATP content of fish and toad embryos. M.S. Thesis. Univ. of Tokyo.

Sarkar, N. K., 1950. Observations on the glycerinated *Limulus* muscle. *Enzymologia,* **14**: 288.

Schneider, C., 1946. Phosphorous compounds in animal tissues. 3. A comparison of methods for the estimation of nucleic acids. *J. Biol. Chem.,* **164**: 747–751.

Snellman, O., and M. Tenow, 1954. A contractile element containing tropomyosin (actotropomyosin). *Biochim. et Biophys. Acta,* **13**: 199–207.

Szent-Györgyi, A., 1945. Studies on muscle. *Acta Physiol. Scand.,* **9**: Suppl. 25.

Szent-Györgyi, A., 1951. Chemistry of muscular contraction. Sec. Edition. Academic Press, Inc., New York.

Tarver, E., and M. F. Morales, 1951. The reaction between actomyosin and various nucleotides and phosphates, as followed by ultraviolet absorption. *J. Cell Comp. Physiol.,* **37**: 235–236.

Tonomura, Y., 1956. Physicochemical study on the actomyosin-adenosine triphosphate system. *J. Res. Inst. for Catalysis* (*Hokkaido Univ.*), **4**: 87–108.

Tonomura, Y., H. Matsumiya and S. Kitagawa, 1957. The effect of ethylenediaminetetraacetic acid on the interaction between actomyosin and adenosine triphosphate. *Biochim. et Biophys. Acta,* **24**: 568–576.

Tonomura, Y., K. Yagi and H. Matsumiya, 1956. Contractile proteins from adductors of *Pecten.* II. Interaction with adenosine triphosphate. *Arch. Biochem. Biophys.,* **64**: 466–479.

Tsao, T. C., P. H. Tan and C. M. Peng, 1956. A comparative, physicochemical study of tropomyosins from different sources. *Scientia Sinica,* **5**: 91–111.

de Villafranca, G. W., 1955. Adenosinetriphosphatase activity of squid mantle muscle (*Loligo pealii*). *Biol. Bull.,* **108**: 113–119.

Weber, H. H., and H. Portzehl, 1952. Muscle contraction and fibrous proteins. *Adv. Protein Chem.,* **7**: 161–252.

Weber, H. H., and H. Portzehl, 1954. The transference of the muscle energy in the contraction cycle. *Progress in Biophysics,* **4**: 60–111.

THE OCCURRENCE OF CHITIN IN THE LOPHOPHORATE PHYLA

LIBBIE H. HYMAN

American Museum of Natural History, New York 24, N. Y.

Knowledge of the occurrence of chitin among animals is imperfect and often contradictory. Zoologists have had the reprehensible habit of applying the name "chitin" to any hardened brown or yellow structure. Chitin is a definite chemical substance whose presence can be demonstrated only by chemical tests and the name should not be applied unless such tests have been performed. The chemical composition of chitin is discussed by Richards (1951).

The expression "lophophorate phyla" is adopted as a convenient collective term for the three phyla that possess a lophophore, namely, Phoronida, Ectoprocta, and Brachiopoda. As I regard the former two classes of Bryozoa, Entoprocta and Ectoprocta, as independent phyla, the name Bryozoa lapses. The three phyla in question are also called tentaculate phyla after Hatschek and by some zoologists are regarded as classes of a single phylum Tentaculata; but the term "tentaculate" appears unfortunate as many other groups are provided with tentacles. I do not consider the union of the three lophophorate groups into one phylum as desirable although close relationship between them is not to be doubted. During the writing of accounts of these three phyla, I became dissatisfied with the available information regarding the occurrence of chitin among them and decided to add to that information.

Fair information of the occurrence of chitin in animals appears in the articles of Wester (1910), Schulze (1924), and Kunike (1925), and in Richards' book (1951). Wester used the chitosan test but Schulze based his statements on the work of his student Kunike who used a method that has been severely criticized. Hence it appears impossible to place any great reliance on the information in the articles of Schulze and Kunike. A valuable and discriminating article on chitin in animals was contributed by Rudall (1955).

The method employed here is the chitosan-iodine color test as given by Campbell (1929). His directions are repeated in Richards (1951) who also gives a long discussion of this and other tests for chitin. Briefly, the material, submerged in potassium hydroxide saturated in distilled water at room temperature, is heated in closed tubes to 160° C. in a glycerin bath and held at this temperature for about 20 minutes. Both at Beaufort and in New York I found that the hydroxide would start to boil at 155° C. The material, alive or preserved, after removal if necessary of adherent foreign material, was washed in tap water followed by distilled water and then dried on filter paper until it no longer wet the paper. Air drying, tried at first, was abandoned when found to leave too much air in the material causing it to float in the alkali. Any residue after the heating in alkali may contain chitin. After washing out the alkali in tap and distilled water, the residue is flooded with the iodine solution whereupon if chitinous it turns brown. The iodine solution is then removed and weak sulphuric acid added whereupon if chitinous the residue turns violet or purple or in case of a strong test deep purplish black. If chitinous the residue will also dissolve in weak acetic acid and a white precipitate will be thrown down from this by addition of weak sulphuric acid. For brevity, the brown-purple color changes will be referred to below as positive color test.

PHORONIDA

Phoronids are soft-bodied vermiform animals that live in a tube of their own secretion to which there are generally adherent sand or rock grains or other foreign material. Wester (1910) reported a negative test with *Phoronis psammophila* but did not state whether he referred to the animal or the tube, presumably the former. Kunike (1925) declared that the "integument" of *Phoronis,* species unspecified, is chitinous. What is meant by integument is not clear but if the surface layer of the body wall is indicated, then Kunike's result was erroneous.

1. *Phoronis pallida.* This is a small delicate species of which some specimens with and without tubes were kindly presented by Dr. Meredith Jones, who had obtained them by dredging in San Francisco Bay. The animals dissolved completely without trace in hot alkali but the delicate tubes remained and gave a strong positive color test.

2. *Phoronopsis viridis.* A large number of tubes containing the worms were generously sent by Colonel Lee Miles who collected them at Bodega Bay, California. The worm of a grayish green color inhabits tubes much longer than itself, usually several inches long. These are erect stiff tubes composed of a secreted parchment-like material covered with adherent rock grains. Piece of tubes and animals removed from the tubes were treated in hot alkali. As before the worms dissolved completely, coloring the alkali green. The tubes, from which most of the adherent grains had fallen off, remained and gave a very strong positive color test. The treated tubes also dissolved in weak acetic acid and a white precipitate appeared in the solution on addition of weak sulphuric acid.

The foregoing results show that the body of phoronids is devoid of chitin but their secreted tubes are composed of chitin.

MARINE ECTOPROCTA

The ectoproct animal inhabits an exoskeletal case of its own secretion to the inner side of which part of its body wall is immovably fixed. The case is termed "zoecium" and the case plus the adherent body wall is called "cystid." The remainder of the animal, termed "polypide," is movable and can be partly extruded from the opening of the cystid (orifice) by a process of evagination. Upon disturbance the polypide is rapidly invaginated into the cystid and is so found in most preserved material.

Wester (1910) reported that the exoskeleton, which he called cuticle, is chitinous in *Zoobotryon verticillatum* (= *pellucidum*), *Flustra foliacea, Flustra carbasea,* and *Bugula turbinata.* The first of these is a ctenostome, the other three cheilostomes. Wester also mentioned a positive test in *"Therma tubulata."* It has proved impossible to ascertain what this is; there appears to be no such generic name as *Therma* in animals. Schulze (1924) listed Bryozoa as chitinous and this statement has been widely quoted but in fact Kunike (1925) did not test any marine ectoprocts. Richards (1951) in his tabulation of the occurrence of chitin in animals incredibly mangled the available data on Bryozoa (page 45). He listed three categories: most Entoprocta, *Bugula, Barentsia* (misspelled); and stated that the part tested was the operculum. But none of these three categories has any operculum; although *Bugula* is a cheilostome it lacks an operculum. Of the forms indicated by Richards as entoprocts, only *Pedicellina* is such; and of course *Barentsia* is also an entoproct. The other forms listed as entoprocts are ectoprocts. Because of a lack of material I did not make any tests on entoprocts about which Wester reported that the cuticle of *Pedicellina* is partly chitinous whereas Kunike obtained a negative result with *Barentsia discreta.*

Work on marine ectoprocts was done during a sojourn in August, 1957, at the Duke University Marine Laboratory at Beaufort, North Carolina. It is a pleasure to acknowledge my indebtedness to Dr. Frank Maturo for collecting and identifying the material. The material was alive at the beginning of the test unless otherwise stated. After return to New York some additional species found preserved in the collections of the American Museum of Natural History were tested.

1. Ctenostomes. The ctenostomatous ectoprocts have membranous exoskeletal cases devoid of calcareous material, borne on running stolons clothed with the same exoskeletal secretion. A number of species were examined.

Amathia convoluta. Only the exoskeleton of zoids and stolons survived the treatment with hot alkali and gave a positive color test, also dissolving in weak acetic acid from which a precipitate was obtained with weak sulphuric acid.

Anguinella palmata. This forms bushy growths covered with a grayish green deposit. Part of this was removed by brushing and some fell off in the hot alkali. The underlying true exoskeleton of zoids and stolons gave a positive color test.

Zoobotryon verticillatum (preserved). The residue after the treatment with hot alkali gave an excellent positive color test, in confirmation of Wester's result, and dissolved in weak acetic acid.

Alcyonidium gelatinosum (preserved). This ctenostome occurs as gelatinous cylindroid growths composed of numerous contiguous zoids. The gelatinous material appears external to the layer corresponding to the exoskeleton of other ctenostomes. In the hot alkali much of the colonies dissolved, including the gelatinous material. The remaining material, appearing as outlines of the zoidal cases, gave a strong positive color test and dissolved in weak acetic acid.

Alcyonidium mytili (preserved). As in the preceding species outlines of the zoidal cases remained after the treatment with alkali, gave a strong positive color test, and also dissolved in weak acetic acid. In both species a white precipitate appeared in this solution on addition of weak sulphuric acid.

2. Cheilostomes. In the cheilostomatous ectoprocts the orifice of the exoskeletal case is displaced ventrally and is closeable by a little exoskeletal lid, the operculum. It happens, however, that an operculum is wanting in *Bugula* and some related genera. The exoskeletal case is generally thick and inflexible, even when devoid of calcareous deposition, and this condition is compensated by the occurrence of a thin ventral area of body wall, termed the "frontal membrane," whose outer layer is also exoskeletal. The first three species listed are devoid of calcareous deposition, the rest have a calcareous layer between the epidermis and the membranous layer of the exoskeleton. No attempt was made to remove the calcareous layer as it did not seem to interfere with the tests.

Aetea anguina. The minute solitary zoids of this species gave an excellent color test throughout the whole exoskeleton.

Bugula neritina. The entire residue from the alkali treatment gave an excellent positive color test but only partly dissolved in weak acetic acid.

Bugula turrita (preserved). The exoskeleton remained practically intact after the alkali treatment and gave an absolutely typical color test. It is impossible to understand the negative result of Richards and Cutkomp (1946) on *Bugula,* presumably this species. The residue also dissolved in weak acetic and the solution gave a precipitate with weak sulphuric.

Schizoporella unicornis. A positive color test was obtained from membranous material around the orifice and from the avicularia.

Thalamoporella gothica. This species fragmented greatly in the hot alkali and the residues gave a poor color test although they dissolved partly in weak acetic acid.

Membranipora tenuis. The zoids of the genus *Membranipora* have a calcareous back and the whole ventral surface consists of a frontal membrane. The membranes hanging to the residues, including the operculum, gave a good positive color test.

Membranipora tuberculata. This gave the same result as the preceding species. The frontal membranes gave an excellent color test and dissolved in acetic acid.

Conopeum commensale. The frontal membranes including the little spines borne on them in this genus and the surface membranes everywhere gave an excellent positive color test.

Hippothoa hyalina. The opercula and some other surface parts were positive in the color tests.

Parasmittina trispinosa. A positive color test was obtained from the avicularia, small areas around the orifice, and the pores of the ovicells.

3. Cyclostomes. The cyclostomatous ectoprocts are heavily calcified, having tubular calcareous cases with terminal orifice and no operculum. Presumably the calcareous material is covered with a cuticle. Only minute fragments of one species were available.

Crisia eburnea. The color test was negative. In the genus *Crisia* the branches are jointed and these joints consist of thickened cuticle without calcareous deposition. These joints dissolved in the hot alkali, hence are not chitinous.

The foregoing results demonstrate that in ctenostomes and non-calcareous cheilostomes the entire exoskeleton consists of chitin. In calcareous cheilostomes, the avicularia, the opercula, the frontal membranes, and some other parts are generally chitinous. The one cyclostome tested gave a negative result but other cyclostomes should be tried.

FRESH-WATER ECTOPROCTS

Fresh-water ectoprocts have a wholly membranous exoskeleton. Only preserved material was available and most of this was kindly furnished by Dr. Mary Rogick. Zander (1897) reported a negative result in *Plumatella* but Kunike (1925) claimed that the "integument" is chitinous in *Plumatella* and *Cristatella.* The jelly of *Pectinatella* contains chitin (Kraepelin, 1887; Morse, 1930). Lerner (1954) found that the float of the statoblasts of *Plumatella* and *Cristatella* is insoluble in alkali and concluded that it is chitinous.

1. Phylactolaemates. This group includes the typical fresh-water ectoprocts with horseshoe lophophore.

Plumatella emarginata. The exoskeleton survived treatment and gave an excellent positive color test, also dissolving in weak acetic acid.

Plumatella repens. A colony with well expanded polypides was found in the collections of the American Museum of Natural History. The polypides dissolved completely in the hot alkali leaving the exoskeletal tubes and contained statoblasts and both were very positive in the color tests.

Fredericella sultana. This species behaved like *Plumatella.* The entire exoskeleton survived the alkali and was highly positive in the color tests, also dissolving in weak acetic acid.

Pectinatella magnifica, statoblasts. Of course the germinal mass dissolved in alkali but the float remained although losing its brown color. Traces of the hooks were left but these disintegrated on washing in water. The float was highly positive.

2. Gymnolaemates. There are some relatives of marine ectoprocts found in fresh water, known by the circular lophophore.

Paludicella articulata. The small colonies were much disrupted by the treatment with alkali but the fragments gave a good color test.

From the foregoing results it is evident that the entire exoskeleton of fresh-water ectoprocts consists of chitin and the same is true of the float of the statoblasts.

BRACHIOPODA

All materials except *Crania* had been preserved in fluid. *Lingula* was purchased from a biological supply house but the other preserved species were kindly supplied by Dr. Francis Stehli.

1. Inarticulates. It is generally recognized that the shells of inarticulate brachiopods except the Craniidae contain a large amount of chitin. This statement rests on positive tests for chitin in *Lingula* by Schmiedeberg (1882), Krükenberg (1885), and Wester (1910). Wester states that the shell of *Lingula* contains a great deal of chitin and that the thick cuticle of the pedicle is also chitinous. Rudall (1955) records that the mantle setae and the cuticle of the pedicle of *Lingula* are "very well-defined examples of beta chitin."

Lingula. In confirmation of previous results pieces of the shell and the thick cuticle of the pedicle surviving the treatment with hot alkali gave a very strong positive color test. The interior tissues of the pedicle, consisting of connective tissue and muscle, dissolved in the hot alkali. The cuticle after the treatment dissolved in weak acetic acid and the solution threw down a white precipitate on addition of weak sulphuric acid. The setae were not recovered.

Discinisca lamellosa. Pieces of shell surviving the hot alkali colored poorly in the iodine solution but gave a strong dark purple response to sulphuric acid. When placed in acetic acid these pieces did not dissolve noticeably but a white precipitate appeared in the solution on addition of weak sulphuric acid. The membrane covering the slot in the ventral valve is also chitinous. Disciniscids lack a pedicle. The mantle setae were not recovered. The apex of the dorsal valve appeared devoid of chitin.

Crania anomala (dry). Although pieces of the shell after the alkali treatment turned slowly brown in iodine they gave no change of color in weak sulphuric acid and did not dissolve in acetic acid. This confirms the accepted opinion that the shell of the Craniidae lacks chitin. The Craniidae have no pedicle.

Chitin is therefore abundantly present in the shell of inarticulate brachiopods except Craniidae and the mantle setae and cuticle of the pedicle are also chitinous.

2. Articulates. The shell of articulates is generally regarded as calcareous without any chitinous component. Wester (1910) obtained a negative result for chitin with an unidentified articulate. Kunike (1925) likewise reported a negative test in "*Terebratula*" (an extinct genus).

Terebratulina retusa (= *caputserpentis*). A negative color test was obtained with the shell of this species.

Terebratalia transversa. Pieces of the shell after the alkali treatment were negative for chitin but fragments of the short pedicle of this form were strongly positive.

Laqueus californianus. This species stands out from the substrate by a neat pedicle of some length. Although the pieces of shell after the alkali treatment were negative for chitin, the surviving outer layer of the pedicle was strongly positive.

It is clear from the available data that the valves of articulate brachiopods are devoid of chitin but the cuticle of the pedicle is chitinous as in lingulids.

CONCLUSION

The distribution of chitin among animals is probably not of phylogenetic significance. Nevertheless it is suggestive that chitin is the common exoskeletal secretion in the three, closely related, lophophorate phyla whereas it appears entirely wanting in another assemblage of related phyla, the deuterostomes (Echinodermata, Hemichordata, Chordata). The total lack of chitin in echinoderms and chordates is well established. In regard to hemichordates no test appears to have been made on enteropneusts but Rudall (1955) testifies that the coenecium of *Rhabdopleura* disintegrates rapidly in hot alkali and gave no evidence of chitin on x-ray examination. The Pogonophora no doubt belong among the deuterostomes and it would be interesting to make a test on their tubes.[1] Whether chaetognaths can be included in the deuterostomes is indeterminable on present evidence, but in this connection it is suggestive to note that their grasping spines are made of chitin. Although Schmidt (1940) obtained a negative result on these spines with the Schulze-Kunike method, I found that they are highly positive by the chitosan test. Preserved specimens of *Sagitta elegans* dissolved completely in the hot alkali except for the grasping spines which gave a strong color reaction. This finding possibly indicates some divergence of chaetognaths from typical deuterostomes.

---

[1] Since the above was written, Professor A. V. Ivanov, of the Academy of Sciences of Leningrad, has kindly informed that the tubes of the Pogonophora have been tested chemically and found to consist of cellulose.

LITERATURE CITED

CAMPBELL, F. L., 1929. The detection and estimation of insect chitin, and the irrelation of chitinization to hardness and pigmentation of the cuticula of the American cockroach, *Periplaneta americana* L. *Ann. Entomol. Soc. America,* 22: 401–426.

Kraepelin, K., 1887. Die deutschen Süsswasserbryozoen. I. Anatomischer-systematischer Teil. *Abhandl. Gebiete Naturwiss. Naturwiss. Verein Hamburg,* 10, no. 9, 168 pp.

Krükenberg, C. F. W., 1885. Über das Vorkommen des Chitins. *Zool. Anz.,* **8**: 412–415.

Kunike, G., 1925. Nachweis und Verbreitung organischer Skeletsubstanzen bei Tieren. *Zeitschr. vergl. Physiol.,* **2**: 233–253.

Lerner, Hedwig, 1954. Zur Kenntnis des Feinbaues der Flottoblastenschalen von *Plumatella repens* (L.) und *Cristatella mucedo* Cuvier. *Ber. Oberhess. Gesell. Natur- und Heilk. Giessen, Naturiss. Abt.,* New ser. **27**: 111–122.

Morse, W., 1930. Chemical constitution of *Pectinatella. Science,* **71**: 265.

Richards, A. Glenn, 1951. The Integument of Arthropods. xvi + 411 pp., University of Minnesota Press, Minneapolis.

Richards, A. G., and L. K. Cutkomb, 1946. Correlation between the possession of a chitinous cuticle and sensitivity to DDT. *Biol. Bull.,* **90**: 97–108.

Rudall, K. M., 1955. The distribution of collagen and chitin. *In*: R. Brown and J. F. Danielli (eds.), Fibrous proteins and their biological significance. *Symposia Soc. Exptl. Biol.,* no. **IX**: 49–71.

Schmidt, W. J., 1940. Zur Morphologie, Polarisationsoptik, und Chemie der Greifhaken von *Sagitta hexaptera. Zeitschr. Morphol. Ökol. Tiere,* **37**: 63–82.

Schmiedeberg, O., 1882. Ueber die chemische Zusammensetzung der Wohnröhren von *Onuphis tubicola* Müll. *Mitt. Zool. Stat. Neapel,* **3**: 373–392.

Schulze, P., 1924. Der Nachweis und die Verbreitung des Chitins mit einem Anhang über das komplizierte Verdauungssystem der Ophyroscoleciden. *Zeitschr. Morphol. Ökol. Tiere,* **2**: 643–666.

Wester, D. H., 1910. Über die Verbreitung und Lokalization des Chitins im Tierreiche. *Zool. Jahrb. Abt. System.,* **28**: 531–558.

Zander, E., 1897. Vergleichende und kritische Untersuchungen zum Verständnisse der Iodreaktion des Chitins. *Arch. Ges. Physiol.,* **66**: 545–573.

Vol. 114, No. 2 April, 1958

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

BIOLOGICAL LIBRARY WOODS HOLE, MASS.

THE DEPENDENCE OF PIGMENT GRANULE MIGRATION ON THE CORTICAL REACTION IN THE EGGS OF ARBACIA PUNCTULATA[1]

ROBERT D. ALLEN AND EDWARD C. ROWE

Biology Department, Princeton University; Zoology Department, University of Michigan; and the Marine Biological Laboratory, Woods Hole, Massachusetts

The migration of pigment granules (echinochrome chromatophores) to the surface of *Arbacia* eggs about 10 minutes after fertilization is a conspicuous event which marks successful initiation of development in this species. These pigment granules are scattered throughout the cytoplasm in the unfertilized egg. Even before fertilization, they move about in the cytoplasm as rapidly as 5 microns per second (Parpart, 1953). Upon fertilization, they migrate to the fertilized egg surface (McClendon, 1909, 1910), which is characterized by the presence of a hyaline layer and fertilization membrane and where a new cortical gel layer will form just beneath the surface.

In sea urchin eggs, elevation of the fertilization membrane is preceded by a wave of cortical granule breakdown (E. N. Harvey, 1911; Moser, 1939). This wave, usually referred to as the cortical reaction, can now be interrupted or blocked by any of three separate methods in order to study the interaction of fertilized and unfertilized cortex on the one hand, with endoplasm on the other (Allen, 1954; Allen and Hagström, 1955a; Hagström and Allen, 1956). The present study was undertaken to determine whether pigment granule migration occurs as a specific interaction between these cytoplasmic granules and fertilized, but not unfertilized egg surface (cf. preliminary note by Allen and Rowe, 1955).

MATERIAL AND METHODS

Arbacia punctulata gametes were obtained by electrical stimulation (E. B. Harvey, 1952). Eggs were deprived of jelly by brief treatment with acid sea water (pH about 5). Insemination was carried out at a sperm concentration of 3×10^6 sperm per ml. and at 20–22° C.

Aliquots of freshly inseminated eggs were transferred at intervals of a few seconds to 0.001% sodium lauryl sulfate in sea water at 30–32° C. for a period of about two minutes, and then were allowed to cool before being washed in sea water.

---

[1] This investigation was supported by Research Grants C-2609 and C-3022 of the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

Copyright © 1958, by the Marine Biological Laboratory

Similar treatment of unfertilized eggs did not visibly affect their early development when they were subsequently inseminated. Fertilized eggs were similarly unaffected by this treatment when it was applied after the cortical reaction changes were complete. A small percentage of the eggs (usually less than 20%) exposed to elevated temperature and detergent during the cortical reaction showed interruption of cortical granule breakdown, restricted membrane elevation and other characteristics of partially-fertilized eggs (Allen, 1954; Allen and Hagström, 1955a; Hagström and Allen, 1956). These eggs were isolated from the others and observed for the pattern of pigment distribution and stage of development in which they were arrested.

Other eggs were inseminated in quartz capillaries; a combination of stretching and warming to about 26° with unfiltered light from the microscope lamp sufficed to interrupt the cortical reaction. These cylindrical, partially-fertilized eggs were also followed for changes in pattern of pigment distribution and for other indications of early development changes.

RESULTS

Whether the pigment granules have migrated to the surface or not is best observed under intense illumination. Fertilized eggs with pigment migration complete exhibit a dense, peripheral, pigmented shell (ring in optical section); on the other hand, eggs with blocked cortical reaction show a brilliant red *zone* on the fertilized surface (Fig. 1); the rest of the cytoplasm contains scattered pigment granules as in the unfertilized egg. Near the red zones on the partially-fertilized eggs the endoplasm lacks pigment granules. Examination with a 50 × water-immersion lens (Leitz) of that portion of the surface to which the pigment migrated shows local loss of cortical granules and the establishment of a hyaline layer and blister-like fertilization membrane. The rest of the egg surface remains re-fertilizable for at least two hours, and a second insemination during this time results in polyspermy.

The migration of pigment granules from an extensive area of endoplasm bordering on the fertilized cortex suggested the recruitment of granules from a considerable distance. Furthermore, the intense color of the pigment at the fertilized surface of a partially-fertilized egg suggested that this surface had "attracted" a greater share of the total supply of pigment granules than an equal amount of surface in a totally fertilized egg. To test this possibility, several eggs were rendered partially fertilized while in the shape of cylinders with rounded ends in a quartz capillary of small diameter. On Figure 2, it is possible to see the regular sub-surface arrangement of pigment granules in the fertilized part, and the scattered distribution in the unfertilized part of the egg furthest from the point of sperm entry. Note, however, the intensely pigmented ring at the border of the unfertilized and fertilized cortex. It can also be seen in this figure that the width of the zone of unfertilized cytoplasm from which pigment granules were recruited was about 26 microns.

In control experiments, a few eggs were observed over the course of the summer, which responded to detergent alone without insemination. These eggs must have become "partially-activated." The fact that pigment migrated to the activated regions of cortex in these eggs indicated that the mere presence of sperm was not a factor in this migration of pigment.

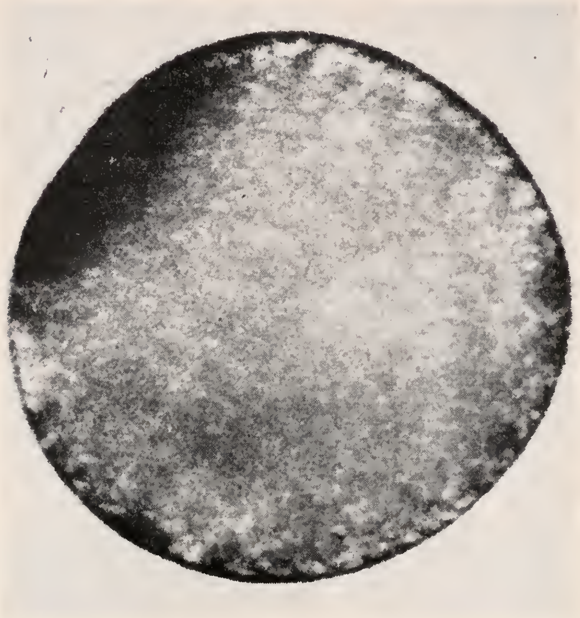


FIGURE 1. A partially-fertilized egg of *Arbacia punctulata* photographed with Kodachrome film and later printed in green light to show the accumulation of pigment (dark) at the region of the egg surface which has undergone a cortical reaction.

Several hundred partially fertilized eggs were observed to determine whether there was any relationship in this species between the amount of cortex affected by the cortical reaction and the extent of development. Such a relationship was clearly evident. Only a few eggs with more than 50% of their surfaces affected by the cortical reaction were observed. (This is probably due in part to the extremely short time that the eggs pass through this condition; *cf*. Figure 1 in Rothschild and

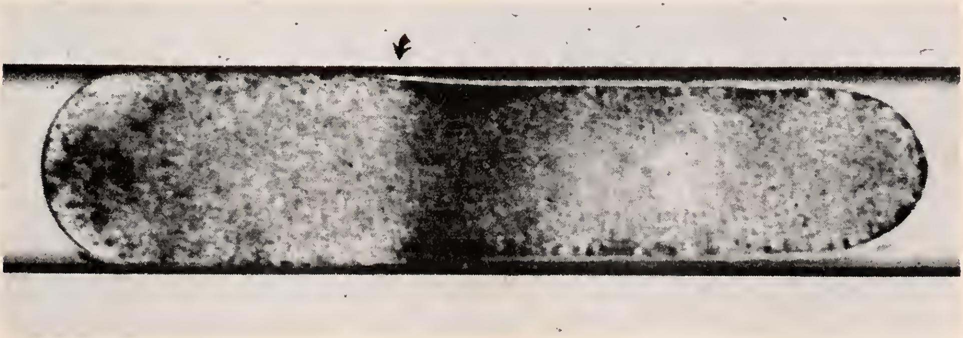


FIGURE 2. Reproduced from Kodachrome as in Figure 1. A partially-fertilized egg obtained by the capillary method (see text). Note the marked accumulation of pigment at the border of fertilized and unfertilized cortex (arrow). This pigment had migrated from nearby unfertilized cytoplasm.

Swann, 1949). Only those eggs with about half of their surface fertilized developed as far as cleavage, and they lacked hyaline layers so that their blastomeres fell apart (*cf.* Herbst, 1900). Eggs with much less than one half their surface fertilized showed varying degrees of development before arrest. The most common stages of arrested development observed after the partial fertilization were metaphase of cleavage, prophase (swollen synkaryon), and varying stages in arrested nuclear migration or fusion. Table I will illustrate by five specific examples the situation often seen in eggs with arrested development showing extremely small amounts of fertilized cortex but containing a sperm nucleus.

TABLE I

Examples showing the relation between the portion of the egg surface affected by the cortical reaction and the extent of development in those eggs.
(*For further description, see text.*)

Percentage of surface area covered by the cortical reaction	Stage in which development was arrested
7.7%	Nuclear migration but no fusion
4.5	Deformed spindle
1.2 (sperm penetrated near egg nucleus)	Nuclei completed copulation path but failed to become centered
1.2 (sperm penetrated 170° from surface nearest egg nucleus)	Sperm nucleus completed copulation path, but no migration of egg nucleus
0.4	Sperm nucleus penetrated 5 microns, no further change in location of nuclei, but nuclear swelling about the time of prophase

DISCUSSION

It is clear from the foregoing evidence that the migration of pigment granules to the cortex after fertilization is the result of an interaction between fertilized cortex and neighboring endoplasm. In previous studies with related species, it was noted that although fertilized and unfertilized cytoplasm become mixed to a significant extent in spherical partially-fertilized eggs, such mixing is very restricted in cylindrical partially-fertilized eggs obtained by the capillary method (Allen, 1954; Allen and Hagström, 1955a; Allen and Hagström, 1955b). These conclusions were based on light-scattering differences which reveal changes in the endoplasm brought about after fertilization. Unfortunately, the presence of pigment in *Arbacia* renders a parallel study with this species impossible. The recruitment of pigment granules from 26 microns deep within cytoplasm that would otherwise be judged unfertilized (Allen and Hagström, 1955a) suggests that the interaction between fertilized cortex and pigment may be more far-reaching than interaction resulting in light-scattering changes.

The mechanism of pigment granule migration under the influence of fertilized cortex remains obscure. The observations of Parpart (1953) with the television microscope suggest that invisible fibrils may extend from the cortex to the individual pigment granules. Such a mechanism would almost necessarily have to be invoked to explain the rapid movement which he observed, especially when this movement sometimes moved perpendicular to the direction of cytoplasmic flow.

Summary

1. The intensely pigmented echinochrome granules (chromatophores) of the *Arbacia* egg, which are distributed throughout the endoplasm prior to fertilization, migrate to the fertilized surface about 10 minutes after insemination.

2. In partially-fertilized eggs, in which the cortical reaction has been blocked or interrupted, the pigment granules migrate only to fertilized cortex.

3. Fertilized cortex in partially-fertilized eggs can recruit pigment granules not only from its immediately-underlying endoplasm, but also from a considerable distance in endoplasm apparently otherwise unaffected by the cortical reaction.

LITERATURE CITED

Allen, R. D., 1954. Fertilization and activation of sea urchin eggs in glass capillaries: I. Membrane elevation and nuclear movements in totally and partially fertilized eggs. *Exp. Cell Research,* **6**: 403–412.

Allen, R. D., and B. Hagström, 1955a. Interruption of the cortical reaction by heat. *Exp. Cell Research,* **9**: 156–167.

Allen, R. D., and B. E. Hagström, 1955b. Some interrelationships among cortical reaction phenomena in the sea urchin egg. *Exp. Cell Research, Suppl.* **3**: 1–15.

Allen, R. D., and E. C. Rowe, 1955. Pigment migration in partially fertilized Arbacia eggs. *Biol. Bull.,* **109**: 344–345.

Hagström, B. E., and R. D. Allen, 1956. The mechanism of nicotin-induced polyspermy. *Exp. Cell Research,* **10**: 14–23.

Hagström, B., and Britt Hagström, 1954. A method of determining the fertilization rate in sea urchins. *Exp. Cell Research,* **6**: 479–484.

Harvey, E. B., 1952. Electrical method of "sexing" Arbacia and obtaining small quantities of eggs. *Biol. Bull.,* **103**: 284.

Harvey, E. N., 1911. Studies on the permeability of cells. *J. Exp. Zool.,* **10**: 507–556.

Herbst, K., 1900. Über das Auseinandergehen von Furchungs- und Gewebezellen in kalkfreiem Medium. *Arch. f. Entw.,* **9**: 424–463.

McClendon, J. F., 1909. On artificial parthenogenesis of the sea urchin egg. *Science,* **30**: 454–455.

McClendon, J. F., 1910. On the dynamics of cell division. II. Changes in permeability of developing eggs to electrolytes. *Amer. J. Physiol.,* **27**: 240–275.

Moser, F., 1939. Studies on a cortical layer response to stimulating agents in the Arbacia egg. I. Response to insemination. *J. Exp. Zool.,* **80**: 423–440.

Parpart, A. K., 1953. The motion of echinochrome granules in the unfertilized egg of *Arbacia punctulata. Biol. Bull.,* **105**: 368.

Rothschild, Lord, and M. M. Swann, 1949. The fertilization reaction in the sea urchin egg. A propagated response to sperm attachment. *J. Exp. Biol.,* **26**(2): 164–176.

CYCLIC CO_2 RELEASE IN INSECTS. IV. A THEORY OF MECHANISM

JOHN BUCK

Laboratory of Physical Biology, National Institutes of Health, Bethesda 14, Md.

I. The Problem

Punt (1944, 1950), while making continuous records of respiration in single insects, using a method involving gas heat capacity ("diaferometer"), discovered that certain species release CO_2 cyclically. In these cycles, periods of very rapid release lasting only a few minutes ("bursts") alternate regularly with periods of very slow release which may last several hours. Subsequent work with the Warburg respirometer (Ito, 1954; Schneiderman and Williams, 1955; Buck and Keister, 1955; Punt, Parser and Kuchlein, 1957) has confirmed Punt's diaferometer measurements and has suggested that the phenomenon may not be as uncommon as might be supposed from the long delay in its discovery. There is, in fact, reason to suspect that the cycling is an exaggeration of respiratory behavior normally occurring in many insects (Buck, 1957; Buck and Keister, 1958).

Though the burst is the more spectacular phase of the cycle, the interburst is the more interesting biophysically, because during this period O_2 is entering the insect through the spiracular valves at several times the rate at which CO_2 is escaping. The present paper attempts to explain how such an unequal gas exchange ratio can exist.

Punt attributed the cyclic CO_2 release to alternate dilation and constriction of the spiracular valves, a view strongly supported by demonstrations on diapausing saturniid pupae that (a) the skin is impervious to gases—*i.e.*, all gas exchange occurs via the spiracles (Schneiderman and Williams, 1955; Buck and Keister, 1955), (b) cycling is abolished reversibly by intubating spiracles, so as to make the tracheal gas continuous with the ambient air, or by making the pupa anoxic, thus forcing the valves to dilate (Buck and Keister, 1955), (c) the spiracles open visibly only at intervals and for periods corresponding, respectively, to burst frequency and duration (Schneiderman, 1956), (d) the spiracles are the only locus in the environment-tissue tracheal pathway where there could be significant physical resistance to gas transfer (Buck and Keister, 1958).

Punt (1944) emphasized the apparent absence of gross ventilatory movements in his insects and found no evidence of rhythmic gas flow, using both Lyonnet's soap bubble test and a horizontal manometer. Changes in total body volume during cyclic respiration were also thought to be excluded in a number of sorts of tests on cecropia pupae (Schneiderman and Williams, 1955) and I have failed to observe any change in linear body dimensions in time-lapse cinematography of a cycling pupa of the moth *Agapema galbina*. From such evidence it has been generally concluded that CO_2 issues from the pupa by diffusion only. Actually, it is possible that ventilatory changes would have been undetectible by the methods used, because it was later found (Buck and Keister, 1958) that total tracheal volume is only of the order of 6% of pupal body volume—which would correspond to an even smaller percentage linear dimensional change, even if the entire content of the tracheae was expelled. However, the low tracheal volume, together with the fact that tracheal pCO_2 is only of the order of 45 mm. Hg, shows that less than 15% of the CO_2 in an average burst could be supplied by gas actually in the tracheae initially (Buck and Keister, 1958). Moreover, ventilation could not be the cause of the fluctuations seen in Warburg respirometer records, unless an actual compression of gas were involved during part of the cycle, because the mere exchange of a given volume of gas between tracheae and environment would not register manometrically.

The small tracheal volume also vitiates the possibility that the spiracles could be sealed during the interburst period, the O_2 uptake that is measured manometrically being supplied wholly from the tracheal gas, with the pupal abdomen telescoping by a corresponding amount (Buck, Keister and Specht, 1953). In *Agapema* (Table I) the amount of tracheal O_2 thus available amounts to only 14–25% of that needed during interburst.

Data on body water content and blood CO_2 capacity in *Agapema* show that the pupa has sufficient stored CO_2 to account for the volume of the burst plus the much larger additional reserve which is present in all phases of the cycle (Buck and Keister, 1958; Buck and Friedman, 1958). These data, therefore, in conjunction with the observed respiratory quotient of about 0.8 for the complete cycle (Table I), and with the evidence for spiracular involvement cited above, argue against the CO_2 bursts being due to any sudden change in metabolic activity of the sort suggested by Zeuthen (1955). Rather, they reinforce the idea that the cycle involves an alternating impoundment and release, by the valves, of the CO_2 produced in steady, normal aerobic respiration.

The presence in the pupa of carbonic anhydrase (Buck and Friedman) makes it reasonable to suppose that the brief burst, during which CO_2 may be given off dozens of times as fast as it escapes between bursts—can be adequately explained as diffusive escape of CO_2 occurring during a period of spiracular dilation. The notably low rate of CO_2 release during the interburst period would seem likewise consistent with spiracular impoundment of CO_2 and its storage in body buffers, for the valves are then so greatly constricted as to appear closed (Schneiderman, 1956), and the tracheal pCO_2 apparently increases, though slowly, throughout the period (Buck and Keister, 1958). However, during this period in which escape of CO_2 is being severely impeded, O_2 appears to be entering the tracheal system freely. For example, the rate of O_2 uptake in Warburg measurements of individual pupae shows no change comparable in magnitude to the burst over periods so long as to certainly include intervals when a burst is occurring and the spiracles are dilated (Schneiderman and Williams, 1955; Buck and Keister, 1955). Likewise, the rate of O_2 uptake is the same in 100% O_2 as in air (Buck and Keister, 1955).

Such a combination of unimpeded O_2 transfer and restricted CO_2 transfer is a serious obstacle to the idea of CO_2 retention by the spiracles, as is easily deduced from the following elementary physical considerations: The rate of gas diffusion through a tube is described by the Fick equation, $R_d = D\ (\pm C_o \mp C_i)\ A/L$, where R_d is the rate of transfer of the particular gas, D its diffusivity, $(\pm C_o \mp C_i)$ its concentration difference between the ends of the tube, and A and L are the cross-sectional area and length, respectively, of the tube. Since, in the present situation.

TABLE I

Measured respiration data for Agapema and Hyalophora

Datum and symbol	Species	Measured parameter	Computational unit
Rate of O_2 uptake per live gram (RO_2)	Agapema 1953[1] Agapema 1954[1] Hyalophora[2]	30 μL/g./hr. 13 μL/g./hr. 14.8 μL/g./hr	5.1×10^{-7}/cm^3/sec./spiracle 2.1×10^{-7}/cm^3/sec./spiracle 14×10^{-7}/cm^3/sec./spiracle
Over-all rate of CO_2 release per live gram (ΣRCO_2)	Agapema 1953[1] Agapema 1954[1] Hyalophora[2]	21.1 μL/g./hr. 10.9 μL/g./hr. 12.5 μL/g./hr.	3.6×10^{-7}/cm^3/sec./spiracle 1.76×10^{-7}/cm^3/sec./spiracle 11.8×10^{-7}/cm^3/sec./spiracle
Interburst rate of CO_2 release per live gram ($IBRCO_2$)	Agapema 1953[1] Agapema 1954[1] Hyalophora[2]	11.9 μL/g./hr. 3.8 μL/g./hr. 2.6 μL/g./hr.	2.0×10^{-7}/cm^3/sec./spiracle 0.6×10^{-7}/cm^3/sec./spiracle 2.46×10^{-7}/cm^3/sec. /spiracle
Mean pupal live weight	Agapema 1953[1] Agapema 1954[1] Hyalophora[2]		0.85 g. 0.81 g. 4.72 g.
Burst volume	Agapema 1953[1] Agapema 1954[1] Hyalophora[2]	33.3 μL/g. 32 μL/g. 57.4 μL/g.	
Mean cycle length	Agapema 1953[1] Agapema 1954[1] Hyalophora[2]	3.6 hr. 4.5 hr. 7.4 hr.	
Respiratory quotient	Agapema 1953[6] Agapema 1954[6] Hyalophora[7]	0.71 0.84 0.85	
Tracheal CO_2 tension (pCO_2)	Agapema[3] Hyalophora[4]	45 mm. Hg (45 mm. Hg)	5.9×10^{-2}/cm^3/cm^3 (5.9×10^{-2}/cm^3/cm^3)
Spiracular valve length (L)	Agapema[3] Hyalophora[5]	50 μ 100 μ	5×10^{-3} cm. 1×10^{-2} cm.
Tracheal volume	Agapema[3] Hyalophora[3]	60 μL/g. 75 μL/g.	

[1] Buck and Keister, 1955. [2] Schneiderman and Williams, 1955. [3] Buck and Keister, 1958. [4] Estimated from blood CO_2 capacity, Buck and Friedman, 1958. [5] Estimate from Dr. W. Beckel. [6] Due to arithmetical error, the values were given by Buck and Keister, 1955 as 0.65 and 0.81 for the two samples, instead of 0.71 and 0.84. [7] Computed from Table I in Schneiderman and Williams, 1955. Elsewhere the value is given as 0.78.

O_2 and CO_2 are passing simultaneously through the same spiracular valves, and since the diffusivities of the two gases differ only in the ratio of 5 : 4, it is clear that any considerable disparity in their diffusive transfer rates would require that there be a corresponding difference in their concentration gradients between tracheae and environment. In *Agapema* pupae, where the interburst pCO_2 is about 45 mm. Hg, the observed average interburst $O_2 : CO_2$ transfer ratio of about 3 : 1 during respiration in air (Table I) might possibly be explained on the basis of relative gradients.

Thus, by assuming a tracheal pO_2 of 20 mm. Hg the gradient ratio would be (Table II) approximately $(155 - 20):(45 - 0)$. However, in the pupa of the cecropia moth, *Hyalophora,* which has about the same relative blood CO_2 capacity as the *Agapema* pupa, disparities in concentration gradients could not possibly explain the observed average $O_2:CO_2$ transfer ratio of 6:1, let alone the ratios of 20:1 or more observed in some individuals. Moreover, even in *Agapema* the requisite partial pressures of O_2 and CO_2 would apparently lead to a badly unbalanced total pressure budget: Atmosphere (Table II) $= 155\ (O_2) + O\ (CO_2) + 23\ (H_2O$ at 25° C.$) + 582\ (N_2) = 760$ mm. Hg; tracheal gases (respectively) $= 20 + 45 + 23 + 582 = 670$ mm. Hg.

In sum, then, the most dramatic feature of the CO_2 release cycle—the burst—seems much less mysterious than the gas exchange during interburst. In this period, as we have seen, the observed degree of CO_2 retention cannot, in the face of unrestricted entry of O_2, be due only to areal reduction in rate of diffusive escape of CO_2. Yet, as we have also seen, diffusion appears to be the only mode of gas transfer involved in the cycle. A possible way of relieving this paradox is suggested in the theory given below.[1]

TABLE II

Physical constants for computations

Constant and symbol	Common unit	Computational unit
Tracheal water tension (sat.) (25°)	23 mm. Hg	$3 \times 10^{-2}/cm^3/cm^3$
Atmospheric O_2 tension (pO_2)	155 mm. Hg	$2 \times 10^{-1}/cm^3/cm^3$
Atmospheric N_2 tension (pN_2)	582 mm. Hg	$7.7 \times 10^{-1}/cm^3/cm^3$
Diffusivity of O_2 (25°) (D_{O_2})		$2.0 \times 10^{-1}/cm^2/sec.$
Diffusivity of N_2 (25°) (D_{N_2})		$2.14 \times 10^{-1}/cm^2/sec.$
Diffusivity of CO_2 (25°) (D_{CO_2})		$1.66 \times 10^{-1}/cm^2/sec.$
Viscosity of air (25°) (η)		1.8×10^{-4}/dyne secs./cm^2
One atmosphere pressure (P)	760 mm. Hg	1.015×10^6/dynes/cm^2

II. OUTLINE OF THEORY

As we have seen, high trans-spiracular $O_2:CO_2$ transfer ratios seem to demand either that in-diffusion of O_2 be supplemented by some non-diffusive process, or that diffusive escape of CO_2 be impeded by some barrier or mechanism in addition to the spiracular valve. The simplest agency for achieving such conditions—and one that achieves both of them simultaneously—appears to be an inward bulk flow of air. This flowing air, because of its 21% O_2 content, would increase specifically the rate of inward transfer of O_2. At the same time the in-flowing O_2 and N_2 molecules of the air, having an excess momentum in the inward direction, would tend to drive back or slow the out-diffusing CO_2 molecules with which they collide.

No in-flow of air could of course occur unless the tracheal gas were at a lower barometric pressure than the outside atmosphere. The "suction" which is visualized as causing the flow is thought to develop as follows: During the burst, when the spiracles are wide open, there must be a major diffusive exchange of tracheal gases with those of the environment, so that when the valves constrict at the end of the burst the tracheal O_2 concentration is higher, and the CO_2 concentration lower, than during the interburst period. The constriction of the valves tends to reduce the rate of diffusive entry of O_2 below the constant rate at which O_2 is being taken up by the tissues, so that tracheal pO_2 falls. (This does not necessarily mean that the tissues ever actually become perceptibly hypoxic—it simply means that a steeper trans-spiracular O_2 gradient has to develop in order to keep the rate of entry of O_2 up to the required respiratory level.) At the same time this drop in intratracheal pO_2 is occurring, a rise in tracheal pCO_2 must begin, due also to the valve constriction, but CO_2, because of its being largely combined with blood and tissue buffers, will actually escape into the tracheae from the tissue liquids at a considerably lower rate than that at which O_2 enters. Hence, during a relatively brief transition period after spiracular constriction, the total intratracheal pressure will fall because O_2 is leaving the gas phase faster than it is being replaced by any gas from either tissues or environment. The air in-flow produced by the suction thus generated impedes the escape of CO_2 and adds to the retention due to the restricted valve. As an extraneous complication, the air flow brings in four volumes of N_2 for every volume of O_2; hence N_2 must accumulate until out-diffusion of N_2 balances in-flow of N_2 and the long interburst period of relatively steady-state gas exchange is begun.

---

[1] A condensed version of the theory was presented before the Tenth International Congress of Entomology in August, 1956, and is in press in the Congress proceedings.

The theoretical interrelations of bulk flow and diffusion, the details of computational methods, and certain other aspects of gas transfer, will be dealt with elsewhere in detail. However it can be said in general that although a steady-state of the sort here postulated has apparently not been analyzed by students of gas kinetics, the analogous but simpler situation of trans-membrane transfer in solution has had some attention (*e.g.*, Jacobs, 1935; Koefoed-Johnson and Ussing, 1953; Garby, 1957) and the possibility of evaluating quantitatively the net transfer resulting from simultaneous and opposed flow and diffusion is provided for by a convection term in the full "diffusion" equation of which Fick's first law is a special case (see, for example, Crank, 1956, pp. 225–228; Jost, 1952, pp. 46–47). I shall refer to the physical process of combined diffusive transport and bulk flow as "flow-diffusion," and its proposed application to respiration as the "flow-diffusion theory."

In view of the present lack of methods for detecting minute bulk flows empirically, the question of whether convective transfer is actually involved in the pupal CO_2 cycle cannot be decided directly but, as shown below, the idea can be tested both for its arithmetic consistency and range of application, and in relation to empirical changes in cycle parameters.

III. Theoretical Interburst O_2, CO_2 and N_2 Exchange Rates

In order to simplify the computational testing of the flow-diffusion theory we shall skip the postulated brief transition period, during which the intratracheal suction develops and in-flowing air builds up tracheal pN_2 and attempt to describe quantitatively the main interburst period, considering it, as a first approximation, to be a steady-state. During interburst the rate of air in-flow must be just sufficient, in conjunction with the restricted spiracular valves, to impound CO_2 at the observed rate, maintain O_2 entry at an adequate level, and bring in N_2 at exactly the rate at which that gas is diffusing outward. As is clear from Table I, there are not enough empirical data to compute the air flow rate directly. My procedure has accordingly been to test various arbitrarily chosen flow rates by successive approximations, until one is found that permits an arithmetical balancing of all the gas exchanges. The computations below define a steady-state during the interburst period for the 1953 sample of *Agapema* (Table I, last column) and illustrate the method. For simplicity everything is expressed on a single spiracle basis, assuming that each member of the 7 pairs is equivalent. Furthermore, the analysis is made in terms of a water-saturated gas, corresponding to the atmosphere of the Warburg flask. The more complex situation where water vapor flux is involved simultaneously will be considered elsewhere in relation to the water-conserving aspects of bulk air in-flow. A number of simplifications have been adopted, such as (a) considering the valve aperture to be circular, (b) ignoring the probability that valve behavior during the burst is not a simple all-or-none dilation and constriction but may involve flutter and a brief period of total closure, (c) taking O_2, N_2 and CO_2 to be equivalent insofar as molecular collision is concerned, and (d) neglecting the "pore-diffusion" correction for valve length.

a. *Observed rate of CO_2 retention.* The total "rate" of CO_2 retention equals the potential rate of release (*i.e.*, the rate of production) minus the observed rate of release during interburst. From Table I this is seen to equal $\Sigma RCO_2 - IBRCO_2 = RET_\Sigma\ CO_2 = 3.6 \times 10^{-7} - 2.0 \times 10^{-7} = 1.6 \times 10^{-7}$ cm³/sec./spiracle.

b. *Postulated air flow rate.* Part of the CO_2 is retained by spiracular constriction (*i.e.*, orifice reduction of out-diffusion) and part by air countercurrent. Numerous trial computations showed that 1.2×10^{-7} cm³/sec./spiracle is the air flow rate ($R_f AIR$) necessary to fit the requirements of the subsequent computational steps. This means, parenthetically, that about 75% of the total measured CO_2 retention is due to countercurrent ($RET_f\ CO_2 = R_f AIR = 1.2 \times 10^{-7}$ cm³/sec./spiracle) and 25% to diffusion limitation by the restricted valve ($RET_a\ CO_2 = 0.4 \times 10^{-7}$ cm³/sec./spiracle).

c. *Potential rate of CO_2 escape by pure diffusion.* By adding the flow-retention rate (Step b) to the observed interburst release rate we obtain the rate at which CO_2 would diffuse out through the spiracular valve if there were no in-flow of air: $RET_f\ CO_2 + IBRCO_2 = R_d CO_2 = 1.2 + 2.0 = 3.2 \times 10^{-7}$ cm³/sec./spiracle.

d. *Interburst spiracular valve area.* Since we know tracheal CO_2 concentration, length of the valve passage and diffusivity of CO_2 (Tables I and II), and potential rate of CO_2 out-diffusion (Step c), we can substitute these values in a rearrangement of the Fick equation and compute the area of the interburst spiracular valve:

$$A = \frac{R_d CO_2 \times L}{D^{CO_2}_{25^\circ}\,(C_i CO_2 - C_o CO_2)} = \frac{(3.2 \times 10^{-7})\,(5 \times 10^{-3})}{(1.66 \times 10^{-1})\,(5.9 \times 10^{-2})}$$

$$= 16.3 \times 10^{-8}\,\text{cm}^2 = 16.3\,\mu^2.$$

This value seems, on the one hand, amply small to account for the closed appearance of the spiracles under the necessarily low magnification employed (Schneiderman, 1956) and, on the other, not unreasonably small in view of the meticulous water vapor conservation which the pupa must exercise during its diapause of many months. There seems, further, no reason to doubt the ability of spiracular valves to constrict to such a minute opening—or even seal entirely, for that matter—in view of the known ability of some insects to resist poisonous vapors such as cyanide for many hours unless the spiracles are forced open by concurrent exposure to CO_2.

e. *Diffusive rate of O_2 uptake.* The over-all measured rate of O_2 uptake (Table I) includes both the O_2 diffusing through the valve, due to the O_2 concentration difference between atmosphere and tracheae, and the 21% of the in-flowing air that is O_2. Hence to obtain the rate at which O_2 should diffuse into the pupa through the restricted spiracular valve if there were no supplementary air in-flow, we subtract 1/5 of the air flow rate from the observed O_2 uptake rate:

$$RO_2 - \frac{R_fAIR}{5} = R_dO_2 = 5.1 \times 10^{-7} - \frac{1.2}{5} \times 10^{-7}$$

$$= 4.86 \times 10^{-7} \text{ cm}^3\text{/sec./spiracle.}$$

Bulk flow of air therefore contributes only 5% to over-all O_2 uptake rate even though it is responsible for much of the CO_2 retention.

f. *Tracheal pO_2.* Knowing, now, the rate of diffusive entry of O_2 (Step e), spiracular valve area (Step d), the diffusivity of O_2 and the valve length (Tables I and II), we can rearrange the Fick equation and solve for O_2 concentration difference between environment and tracheae:

$$C_o^{O_2} - C_i^{O_2} = \frac{R_dO_2L}{D_{O_2}A} = \frac{(4.86 \times 10^{-7})\ (5 \times 10^{-3})}{(2.0 \times 10^{-1})\ (16.3 \times 10^{-8})} = 7.4 \times 10^{-2} \text{ cm}^3\text{/cm}^3,$$

which is approximately 56 mm. in partial pressure units. Subtracting this figure from 155 mm., the partial pressure of O_2 in the (water-saturated) atmosphere gives 99 mm. Hg as intratracheal pO_2.

g. *Tracheal pN_2.* Knowing tracheal pO_2 (Step f), pCO_2 and pH_2O (Tables I and II), the pN_2 required to maintain total pressure balance is readily obtained by summing the other partial pressures and subtracting from 760:760 − (99 + 45 + 23) = 593 mm. Hg. An outward N_2 partial pressure difference between tracheae and environment of about 11 mm. Hg (593 − 582) should therefore exist during the interburst period ($= 1.45 \times 10^{-2}$ cm³/cm³). It may appear contradictory to assume that total tracheal pressure is 760 mm. Hg when the basic driving force of the postulated flow is a difference in total pressure between tracheae and ambient gas. However, preliminary estimates show that the total pressure differential required is considerably less than 1 mm. Hg (see also Step i, below).

h. *Balance of N_2 in-flow and out-diffusion.* From the values for tracheal pN_2 (step g), valve area (step d), valve length (Table I) and diffusivity of N_2 (Table I), the potential rate of out-diffusion of N_2 is computed:

$$R_dN_2 = \frac{D_{N_2}(C_i^{N_2} - C_o^{N_2})A}{L} = \frac{(2.14 \times 10^{-1})\ (1.45 \times 10^{-2})\ (16.3 \times 10^{-8})}{5 \times 10^{-3}}$$

$$= 1 \times 10^{-7} \text{ cm}^3\text{/sec./spiracle.}$$

The rate of N_2 in-flow is of course 4/5 of the total air flow rate, or 0.96×10^{-7} cm³/sec./spiracle. Hence the theoretical requirement of equilibrium between in-flowing and out-diffusing N_2 is satisfied by an air flow rate of 1.2×10^{-7} cm³/sec./spiracle. The attainment of an arithmetical balance indicates that the values for the other parameters computed on the basis of this flow rate are mutually compatible.

i. *Total pressure differential.* The rate of laminar flow through a cylindrical tube is given by Poiseuille's equation, $R_f = P\pi r^4/8\ L\eta$, where P is the barometric pressure difference between the ends of the tube, r the radius and L the length of the tube, and η the viscosity of the flowing medium. By rearranging the equation and substituting our chosen R_f value (Step b), the radius which would correspond to the valve area of 16.3×10^{-8} cm² (Step d), and other values from Table I, we have:

$$P = \frac{8L\eta R_f}{\pi r^4} = \frac{8\ (5 \times 10^{-3})\ (180 \times 10^{-6})\ (8.25 \times 10^{-8})}{3.14\ (2.28 \times 10^{-4})^4}$$

$$= 110 \text{ dynes/cm}^2 = 2.1 \times 10^{-4} \text{ atmospheres} = 0.08 \text{ mm. Hg.}$$

An astonishingly small pressure head, therefore, in conjunction with a spiracular valve of 16.3 μ^2 area and the observed rate of O_2 uptake, is sufficient to maintain the air flow required to bring about the observed CO_2 retention in *Agapema.*

In sum, it is possible to set up a mainly hypothetical but internally consistent balance of O_2, CO_2 and N_2 transfers that accounts for the observed CO_2 retention during the interburst period of the pupal respiratory cycle in *Agapema.*

IV. THEORY IN RELATION TO POSSIBLE DEGREE OF CO_2 RETENTION

The computations set forth in Section III have demonstrated the possibility that an approximately 3 : 1 O_2 uptake/CO_2 release ratio could be induced by a combina-

TABLE III

Computed steady-state values for respiratory parameters fitting various observed and hypothetical degrees of CO_2 retention in Agapema and Hyalophora

RO_2 (cm³ × 10⁻⁷/ sec./spir.)	ΣRCO_2 (cm³ × 10⁻⁷/ sec./spir.)	$IBRCO_2$ (cm³ × 10⁻⁷/ sec./spir.)	$\frac{RO_2}{IBRCO_2}$	R_dCO_2 (cm³ × 10⁻⁷/ sec./spir.)	Σ_{ret} (cm³ × 10⁻⁷/ sec./spir.)	Postul. R_f AIR (cm³ × 10⁻⁷/ sec./spir.)	Valve area (cm² × 10⁻⁸)	Tracheal pO_2 (mm. Hg)	Tracheal pN_2 (mm. Hg)	R_dN_2 (cm³ × 10⁻⁷/ sec./spir.)	R_fN_2 (cm³ × 10⁻⁷/ sec./spir.)	P (mm. Hg)	% CO_2 retained
1953 Agapema													
5.1	3.6	2.0	2.5	3.2	1.6	1.2	16.3	99	593	1.0	0.96	0.08	45
5.1	3.6	0.5	10	2.6	3.1	2.1	13.3	88	604	1.64	1.68	0.2	86
5.1	3.6	0.25	20	2.45	3.35	2.2	12.8	86	606	1.73	1.76	0.23	93
1954 Agapema													
2.1	1.76	0.6	3.5	1.23	1.2	0.63	6.3	96	596	0.5	0.5	0.27	68
2.1	1.76	0.2	10	1.06	1.56	0.86	5.4	87	605	0.69	0.69	0.51	89
2.1	1.76	0.1	20	1.02	1.66	0.92	5.2	85	607	0.73	0.74	0.59	94
Hyalophora													
14	11.8	2.46	5.7	7.66	9.34	5.2	78	92	600	3.9	4.1	0.03	79
14	11.8	1.4	10	7.2	10.4	5.8	73	88	604	4.5	4.6	0.038	88
14	11.8	0.7	20	6.9	11.1	6.2	70	85	607	4.9	5.0	0.043	94

tion of spiracular constriction and in-flow of air, without limiting the rate of O_2 uptake. Similar serial computations indicate the theoretical feasibility of widely different degrees of relative CO_2 retention, both in systems with several parameters the same as in *Agapema,* and in systems involving known respiratory data from *Hyalophora* (Table III).

V. THEORY IN RELATION TO INDUCED CHANGES IN INTERBURST CO_2 RELEASE RATE

In response to certain environmental and endogenous alterations the rate of CO_2 release during the interburst period undergoes definite and reproducible changes. These must be accounted for by any theory purporting to explain cyclic CO_2 release. Of these, all that involve a reduction in demand for O_2 (Table IV, responses No. 2, 4), or increase in availability of O_2 (No. 5), decrease the rate of CO_2 release; and all tending to decrease the availability of O_2 (No. 6), or increase the call for O_2 (Nos. 1, 3), have the opposite effect.

TABLE IV

Effects of certain environmental and endogenous changes on rate of CO_2 release during the interburst period

Response No.	Change	IBRCO₂
1	Increasing temperature	Increase[1,2,3]
2	Decreasing temperature	Decrease[1,2,3,5]
3	Increasing metabolic rate (injury; dev.)	Increase[3]
4	Decreasing metabolic rate	Decrease[2,3]
5	Increased ambient pO_2 above 21%	Decrease[3,4]
6	Decreased ambient pO_2 (below 21%, but above about 10%)	Increase[3,4]

[1] Punt, 1944.
[2] Punt, 1950.
[3] Schneiderman and Williams, 1955.
[4] Buck and Keister, 1955.
[5] Buck and Keister, 1958.

Now we know (Buck and Keister, 1955) that environmental O_2 concentrations between 1% and 100% neither increase nor decrease rate of O_2 consumption (CO_2 production), so the observed changes in CO_2 release rate must be due to changes in rate of CO_2 retention. The retention changes in turn must involve changes in air in-flow and CO_2 out-diffusion due to alterations in either spiracular valve area or effective CO_2 gradient, or both. Although no direct measurements of spiracular valve areas in pupae with different metabolic rates or in different O_2 tensions or temperatures have been reported, the fact that increase in ambient O_2 concentration above the normal atmospheric level reduces the rate of interburst CO_2 release (Table IV, resp. No. 5) without increasing the rate of O_2 uptake, suggests that the maintained, steady-state valve area of the interburst period is regulated so as to keep the rate of entry of O_2 at the minimum that will fully supply respiration (see also Buck and Keister, 1955, 1958; Schneiderman, 1956). A teleological support for this simplifying assumption is the fact that such regulation would also minimize water loss, a particularly acute problem in diapausing pupae since they are denied water intake for many months, and hence would be in line with a main and well established function of spiracles (*e.g.*, Hazelhoff, 1926). The assumption is also compatible with flow-diffusion, and in fact appears to be the only way of reconciling the role of O_2 uptake in inducing air flow with the fact that the rate of O_2 uptake is not O_2-limited except at very low ambient concentrations.

Even for responses not involving changed metabolic rate the expected changes in CO_2 are not easy to predict. For example, insofar as purely diffusive transfer is concerned, a decrease in ambient pO_2, since it induces spiracular dilation, would be expected to increase the interburst rate of CO_2 escape (see Fick equation). However, if the pupa regulates valve area so as to keep the rate of O_2 entry constant, ambient pO_2 will be inversely proportional to area and the effects of changing pO_2 on interburst CO_2 out-diffusion rate will be similarly non-linear (Buck and Keister, 1955, p. 160). On the same basis changing ambient pO_2 will have an inversely proportional effect on R_F (*i.e.* on CO_2 flow-retention), but with the additional complication of a different proportionality constant ($R_D \sim A$, whereas $R_F \sim A \times r^2$; see Poiseuille equation). Hence the over-all effect of changing ambient pO_2 in a flow-diffusion system is not immediately obvious, either qualitatively or quantitatively.

The interrelated changes in valve area, air-flow rate, interburst CO_2 release rate and areal retention rate can be visualized by constructing a family of curves relating valve area and air-flow rate at different pressures (computed from the Poiseuille equation). Figure 1 shows such a plot, to which have been added (a) the straight line relating diffusion rate of CO_2 (R_dCO_2) to valve area for the valve length and trans-spiracular CO_2 gradient of *Agapema,* and (b) the fitted values of air-flow rate and valve area for the respiratory parameters measured in the 1953 and 1954 samples of *Agapema* and for several hypothetical situations involving greater degrees of CO_2 retention (Table III). Figure 2 is a similar plot, with different isobars and R_d line, for cecropia. To illustrate the use of the isobar plot, let us consider the point 6.3, 0.63 in Figure 1, which represents the values computed for the observed gas exchange of the 1954 sample of *Agapema* pupae (RO_2 to $IBRCO_2$ ratio of 3.5 : 1, Table III). The distance from the Y or transfer-rate axis to this point is of course the valve area of 6.3×10^{-8} cm² or 6.3 μ^2. The distance from the X or valve area axis to the point is the rate of retention caused by airflow (0.63×10^{-7} cm³/sec.). The vertical distance from the point to the R_d line is the rate of CO_2 release during the interburst period ($R_dCO_2 - R_fAIR = IBRCO_2 = 1.25 - 0.63 = 0.62 \times 10^{-7}$ cm³/sec.). The further distance on the 6.3 ordinate to the horizontal broken line at 1.76 (which represents the over-all rate of CO_2 *production* by these pupae per spiracle) is the areal retention (RET_a) or the fraction which could theoretically be retained by valve constriction alone—*i.e.*, the reduction in potential diffusive loss – ($\Sigma RCO_2 - R_dCO_2 = RET_aCO_2 = 1.76 - 1.25 = 0.51 \times 10^{-7}$ cm³/sec.).

Considering now the theoretical effects of decreased ambient pO_2 on the 1954 *Agapema* pupae as a concrete test of the flow-diffusion theory, we know that spiracular valve area must increase if O_2 uptake rate is to be maintained. The isobar plot shows us that all combinations of air-flow rates and valve areas that fit the measured respiratory parameters of the 1954 pupae lie along the line AB. Any increase in valve area, therefore, should call for decreased R_f, P and areal retention, and therefore for an increased interburst rate of CO_2 release. Specifically, if we imagine area to increase to 8 μ^2 (line CD) and assume that the R_d line is constant (triggering pCO_2 remains at 45 mm.), R_f will fall to about 0.2×10^{-7} cm^3/sec., interburst rate will increase to $1.57 - 0.2 = 1.37 \times 10^{-7}$ cm^3/sec., RET_a will decrease from 0.51 to about 0.18 cm^3/sec. and P will fall from 0.255 to about 0.052 mm. Hg. Clearly also, an *in*crease in ambient pO_2 (inducing a decrease in valve area) would increase CO_2 retention by both increased P (air-flow) and by areal restriction. Insofar as interburst rate is concerned, therefore, the theoretical changes are in the observed directions.

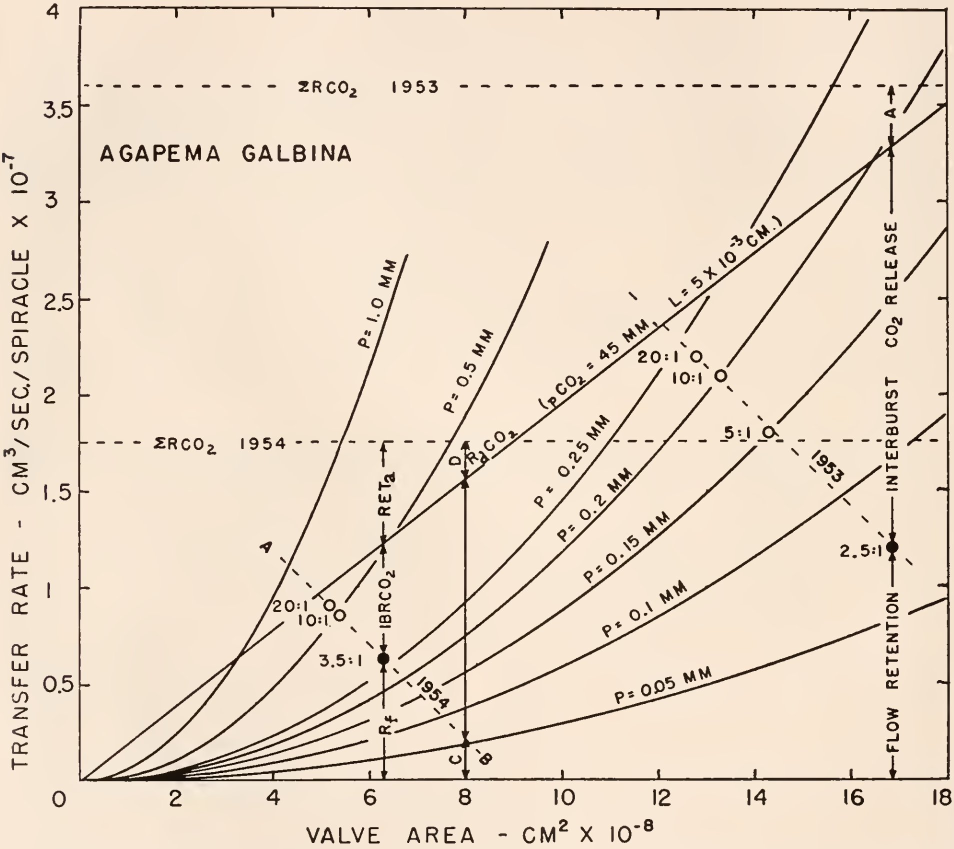


FIGURE 1. Relation between valve area (abscissa) and gas transfer rate (ordinate) at different barometric pressure deficits (curved lines). The two horizontal broken lines mark the over-all rates of CO_2 production in two samples of *Agapema* pupae. The diagonal broken lines connect points representing various ratios of O_2 uptake rate to interburst CO_2 release rate, the two solid points representing the values computed for the actual measurements for the two samples, and the hollow circles representing hypothetical values (Table III). Additional exposition in text.

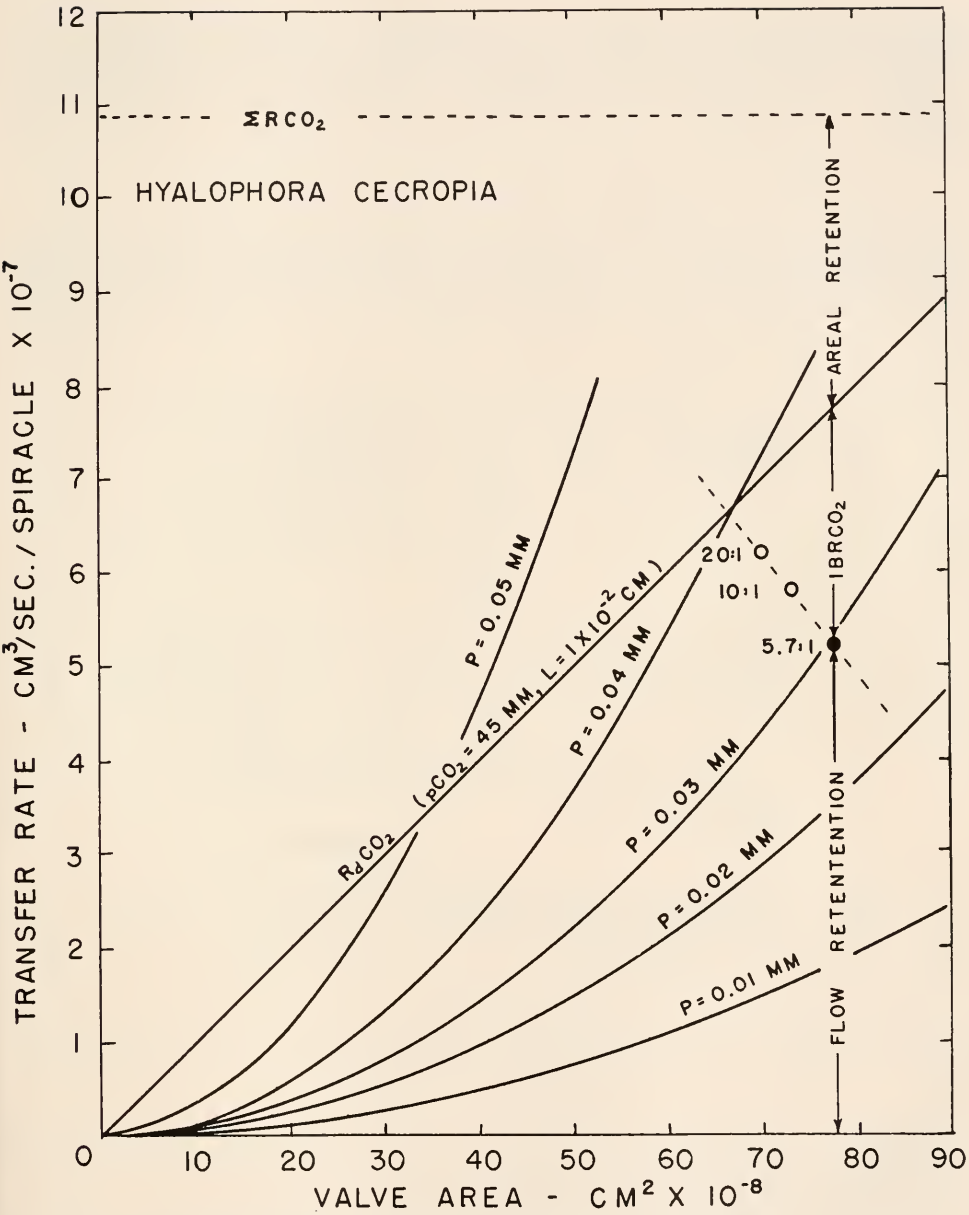


FIGURE 2. Plot corresponding to Figure 1, for *Hyalophora.*

When changes in both metabolic rate and valve area occur (Responses No. 1–4, Table IV) the consequences are too complex to predict with any assurance. Thus, if we assume a change involving an increased metabolic rate it appears that valve area would have to increase, but it seems by no means excluded that part of the extra O_2 needed could be supplied by steepening the O_2 concentration gradient in addition. Furthermore there is no assurance that the R_d line would remain unchanged. Accordingly, the best that can be said is that the observed increase in interburst leakage with increasing temperature and metabolic rate is allowed by the theory.

VI. Theory in Relation to Complete Cycle

Though the flow-diffusion idea has been applied primarily to the CO_2 retention of the interburst period, it is necessary to be certain that burst production is also provided for in the theory and that the two phases of the cycle are physically and biochemically compatible. Furthermore the theory must account for any changes observed in O_2 uptake rate or O_2 tension.

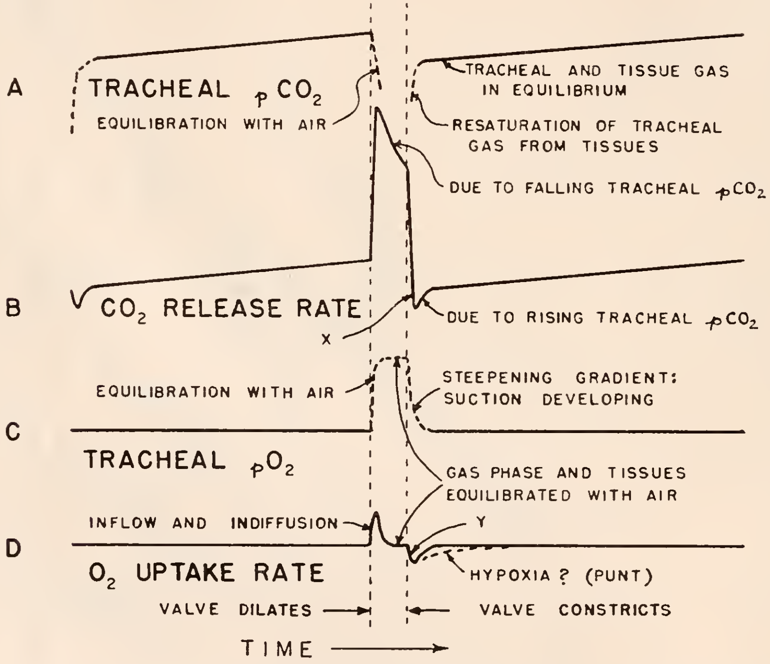

A
TRACHEAL pCO_2
EQUILIBRATION WITH AIR
TRACHEAL AND TISSUE GAS IN EQUILIBRIUM
RESATURATION OF TRACHEAL GAS FROM TISSUES
DUE TO FALLING TRACHEAL pCO_2
B
CO_2 RELEASE RATE
X
DUE TO RISING TRACHEAL pCO_2
EQUILIBRATION WITH AIR
STEEPENING GRADIENT: SUCTION DEVELOPING
C
TRACHEAL pO_2
GAS PHASE AND TISSUES EQUILIBRATED WITH AIR
INFLOW AND INDIFFUSION
Y
D
O_2 UPTAKE RATE
HYPOXIA ? (PUNT)
VALVE DILATES
VALVE CONSTRICTS
TIME

Figure 3. Time course of various respiratory parameters according to flow-retention theory (diagrammatic). See text.

Figure 3 is a diagrammatic representation of the time courses of O_2 and CO_2 exchange, and of changes in pO_2 and pCO_2, based on Warburg measurements on *Agapema* and *Hyalophora* and on theoretical considerations given below. Though the transfer curves are almost exactly the same as some actual diaferometer records of Punt for *Carabus,* Punt's explanations of certain phases of the exchange differ from mine and have to be reconciled before the theory can be said to be supported by both methods of respirometry.

A. Time course of CO_2 release

Considering first the CO_2 predictions, the decline in rate of CO_2 release which occurs during the burst and *before* valve constriction (Fig. 3B) might be expected on the basis of two findings of Buck and Keister (1958) on *Agapema.* First, some decline would be expected merely on the basis that tracheal pCO_2 has reached about 45 mm. Hg by the end of one interburst period yet has fallen at least to 38 mm. by an early stage in the next. Second, the fact that less than 15% of the total burst volume can be present initially in the tracheal gas phase suggests that the burst might exhibit an almost instantaneous peaking of CO_2 output rate, representing the exit of much of the tracheal (gaseous) CO_2, which then declines as the more slowly released CO_2 from blood and tissues comes to predominate. This implies that tracheal pCO_2 may briefly fall below 38 mm. during the burst, as indicated by the broken part of the curve in Fig. 3A. Indeed this might be predicted from the fact that during the interburst period essentially the entire concentration difference between atmospheric and tracheal gases occurs across the spiracular valve (Buck and Keister, 1958), whereas during the burst, when the valve is widely dilated, the tracheal gas presumably equilibrates more thoroughly with ambient gas and the steepest rise in pCO_2 and fall in pO_2 should occur in the distal tracheae much closer to the actual tissues; or at least the environment-tissue gradients should be much less steep than with a constricted valve. If, then, the spiracles constrict suddenly, there will be a delay in reaching the rate of CO_2 release characteristic of interburst, because tracheal gas must come into diffusion equilibrium with the (higher) blood CO_2 concentration before the steady-state interburst trans-spiracular out-diffusion gradient can be established. Hence there should occur a post-constriction dip in the CO_2 release record (X, Fig. 3B). Punt, Parser and Kuchlein (1957), however, have a different explanation for the post-constriction dip which necessitates a critical consideration of the course of O_2 uptake.

B. Time course of O_2 uptake

When the O_2 uptake rates of diapausing pupae showing cyclic CO_2 release were first measured (Schneiderman and Williams) it was emphasized that there were no disturbances in the manometric records corresponding to the CO_2 burst periods. In most of our Warburg records, likewise, no irregularities exceeding the experimental variation between successive 5-minute readings were observed (Buck and Keister, 1955; see also Fig. 4A in this paper). In a few records, however, we did encounter statistically significant perturbations consisting sometimes of a small apparent excess O_2 consumption (first asterisk, Fig. 4B), sometimes of a small apparent deficiency in O_2 consumption (second asterisk, Fig. 4B) and, rarely, of a small diphasic episode (Fig. 4C). We interpreted all these irregularities as artifacts due to inability of the flask alkali to absorb instantaneously the large volume of CO_2 suddenly released in the burst. Thus a manometer read soon after a burst began, would, if CO_2 were leaving the pupa faster than it could be absorbed, appear to indicate a decrease in the otherwise steady rate of O_2 uptake, or even an apparent net production of gas (downward spikes in Figs. 4B and 4C). Conversely, if the manometer were read after the rate of CO_2 absorption by the alkali had "caught up" with the release rate of CO_2 of the burst, the rapid scavenging of the residual extra CO_2 would be added to the continuing O_2 uptake to give a spurious peak.

In agreement with this idea, the phase of apparently decreased uptake invariably *precedes* the peak of apparently increased O_2 uptake in instances where both supposed artifacts occur together.

Punt (1956) and Punt, Parser and Kuchlein (1957) have recently reported that small spikes, synchronous with CO_2 bursts and sometimes diphasic, are a regular feature of O_2 uptake in several species of insects, particularly carabid beetles, in both manometric and diaferometric records.[2] For the spikes in their Warburg records the Dutch workers have given the same explanation as we—that they are artifacts of equilibration—but for the diaferometer records, in which a peak of apparently depressed O_2 uptake *follows* the peak of increased uptake (Fig. 3D), they propose the quite different explanation discussed below.

Punt and I agree that a rapid fall in tracheal pO_2, during which the tracheal gas is depleted of O_2 down to tissue level, should follow spiracular constriction (Fig. 3C) and that a rapid entry of O_2 should occur when the spiracles dilate at burst time and the tracheal pO_2 rises again to or near atmospheric level (Fig. 3D).[3]

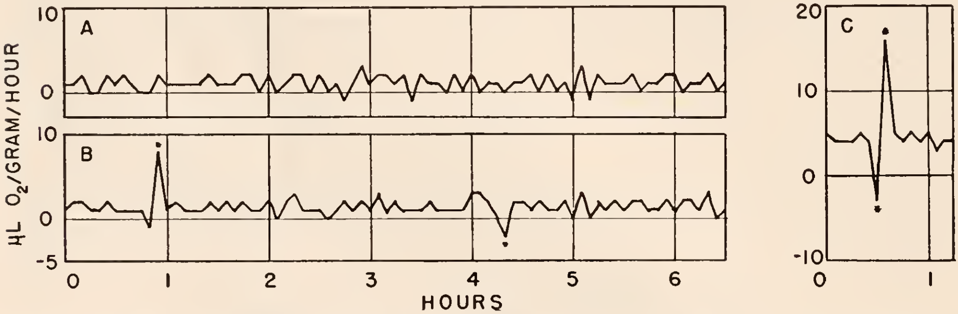


FIGURE 4. Rate of O_2 release in *Agapema* (Figs. 4A, B) and *Rothschildia orizaba* (Fig. 4C) to show non-significant (A) and significant (B, C, asterisks) fluctuations. See also text.

It is also agreed that the falling phase of the down-spike just after spiracular constriction (Y, Fig. 3D) represents the period when tracheal O_2 is passing into the tissues faster than it is entering through the spiracles (*i.e.,* the period when, according to flow-diffusion theory, the increase in trans-spiracular in-diffusion gradient does not keep pace with the decrease in valve area, and the over-all intratracheal pressure deficit is established). Ostensibly these quantitative features of the O_2 record seem readily explicable on physical grounds. Though the computed interburst trans-spiracular gradients of O_2, and the measured gradient of CO_2 in *Agapema,* are approximately equal (56–59 mm. and 45 mm., respectively; Tables II and III), the fact that the CO_2 capacity of the pupal blood is of the order of 55 times that for O_2 (Buck and Friedman, 1958) makes it easy to understand (a) why the initial O_2 in-flow and in-diffusion spike (Fig. 3D), representing the volume of O_2 necessary to re-equilibrate the tracheal space and tissues with atmospheric O_2, is so small in comparison with the CO_2 out-diffusion spike (burst, Fig. 3B), (b) why this O_2 spike would be submerged entirely in most Warburg records (Fig. 4A), and (c) why the rate of O_2 entry falls back to plateau level *before* the spiracle constricts.[4] However, Punt believes that spiracular constriction ("closure") actually reduces respiration to the point where lactic acid accumulates, and that the increasing acidosis causes the interburst rise in tracheal pCO_2 (Fig. 3A). He sees evidence of O_2 debt in *Carabus* in a supposedly lower rate of O_2 uptake during interburst than during the burst (dotted line, Fig. 3D), and apparently regards the initial up-spike of O_2 uptake as going in part to pay off the debt. The question of whether the pupa does actually go into debt does not bear critically on whether bulk flow is involved in pupal gas exchange, but the matter merits further attention, both because of the intrinsic interest of the possibility of an organism making itself hypoxic in *normal* respiration, and in relation to the biochemical aspects of CO_2 retention and release.

---

[2] Heller (1930), in manometric records of O_2 uptake in diapausing *Deilephila* pupae, found that (translation) "Gas exchange took place not constantly, but at intervals whose length was inversely proportional to the intensity of metabolism. It appeared as if the stigmata were tightly closed and then opened at a definite CO_2-tension in the tracheae."

[3] According to flow-diffusion theory the sudden entry of O_2 should also involve at least a slight bulk in-flow component, before the minute total pressure deficit in the tracheae is equalized, and there should also occur a transitory and volumetrically minor out-diffusion of N_2. However these transfers would not be separable from diffusive O_2 uptake by either method of recording.

Punt, Parser and Kuchlein's O_2 debt hypothesis appears to be questionable on the following observational and theoretical grounds: (1) The depressed interburst O_2 uptake to which they refer (Fig. 3D) shows, in my opinion, in only a minority of their *Carabus* recordings, is most marked at the beginning of interburst instead of at the end, and is lacking entirely in their cecropia records. (2) Since neither diaferometer nor Warburg respirometer distinguishes between true respiration and physicochemical equilibration, a change in over-all O_2 or CO_2 exchange rate need not reflect accurately the rate of true tissue oxidations. Thus during a temporary fall in "O_2 uptake rate" (Y, Fig. 3D) the tracheal supply might very well suffice to keep the tissue fully oxygenated until the gradient had steepened sufficiently to permit all metabolic needs to be met by steady-state in-diffusion or by in-diffusion supplemented by in-flow. Similarly, as discussed above, the transient dip in CO_2 release rate after spiracular constriction (X, Fig. 3B) can reasonably be ascribed to a temporarily low tracheal pCO_2 rather than to a depression in the actual rate of CO_2 production. (3) The mere impounding of CO_2 during interburst must lead to some reduction in alkaline reserve. Hence an increase in blood or tissue acidity, even if it were demonstrable, could well be the effect of a rise in pCO_2 rather than its cause. In any case acidosis would be no proof that hypoxia existed. (4) In view of the fact that in many insects (including *Agapema*) O_2 uptake rate does not begin to be O_2-limited until ambient O_2 concentration has fallen to 1% or lower, it seems hardly reasonable that hypoxia can be a factor in cyclic respiration. Surely if interburst tracheal O_2 concentration is anything like the 96–99 mm. Hg (13%) estimated for *Agapema* on theory (Table III), an O_2 debt could not possibly develop. Furthermore, as we have seen, the responses of both *Agapema* and *Hyalophora* to changes in temperature, metabolic rate and ambient pO_2 are consistent with the idea that the valve area is carefully regulated so as to provide an adequate O_2 supply throughout the interburst period. Other evidence in fact indicates that only where O_2 supply is high relative to demand can a cyclic type of CO_2 release occur (Buck and Keister, 1958).

---

[4] The fact that neither dip (X or Y) appears in Punt, Parser and Kuchlein's diaferometer records from the cecropia pupa (their Figs. 6 and 7) may mean that in this species the valve constricts gradually, or flutters, so that the rate of exchange never falls much below the interburst plateau level.

Burst frequency *in*creases under conditions in which the rate of interburst CO_2 release *in*creases (Schneiderman and Williams, 1955; Buck and Keister, 1955) whereas it ought seemingly to require a longer, not a shorter, time for the valve-triggering CO_2 concentration to be attained. This fact is explained by Punt, Parser and Kuchlein by saying that the conditions inducing an increased interburst release rate (low ambient pO_2, increased temperature or metabolic rate) make the O_2 debt develop more rapidly, so that tracheal pCO_2 reaches the triggering level sooner. However, Schneiderman and Williams found interburst release rate to increase more between 10° and 25° than did metabolic rate, so it is difficult to see how CO_2 could accumulate in the tracheae *faster* at high than at low metabolic rate. Probably the paradox is better explained on the assumption that the triggering pCO_2 falls with falling ambient pO_2 (or falling O_2 supply relative to demand). This would agree well with direct observations on spiracular opening in adult insects (Wigglesworth, 1953; Case, 1956), and with the conclusion reached by Schneiderman and Williams on the basis of the fact that burst volume is greater at lower temperatures.

C. The triggering of the burst

Two phenomena are invariably associated at the burst: spiracular valve dilation and sudden increase in rate of CO_2 evolution. It has been generally assumed that the valve dilation is the cause of the burst and that the accumulating CO_2 of interburst is the agent that induces the dilation (Buck and Keister, 1955, 1958; Schneiderman, 1956; Buck, 1957). However, the buffering of CO_2 in insect blood follows the conventional pattern in which most of the H_2CO_3 formed by hydration of free CO_2 combines with base to form bicarbonate ($BHCO_3$).[5] Since acidification of such a liquid phase would obviously increase the concentration of free CO_2, the possibility needs to be considered that the primary cause of the burst is a production of hydrogen ions in blood or tissues, spiracular opening also being due, directly or indirectly, to the same cause. Conversely, if this possibility should prove untenable, an explanation must be given of how mere dilation of the valve could trigger directly the escape of CO_2 from body buffers in addition to liberating the CO_2 in the tracheal gas.

If generation of hydrogen ions were the immediate trigger of the burst, two possibilities might exist: (1) that the rate of release of CO_2 from buffers increases suddenly and markedly when a certain triggering hydrogen ion concentration is attained, or (2) that the rate of appearance of hydrogen ion suddenly increases. The first possibility is negated by the flatness of the curves relating pupal blood

---

[5] Bishop (1923) and Levenbook (1950) found the value of about 6.1 for the first dissociation constant of carbonic acid in the larval bloods of the honeybee and of *Gastrophilus*, respectively, at 16–25° C. If this value is assumed for *Agapema* pupal blood, the Henderson-Hasselbalch equation shows that about 70% of the CO_2 should be in the form of bicarbonate at the normal blood hydrogen ion concentration of about pH 6.45. The $BHCO_3/CO_2$ ratio is presumably still higher in the tissues, but in any case, since less than 15% of the CO_2 in the burst could come from gas initially in the tracheae, it is clear that a major portion of the CO_2 released during the burst must come from bicarbonate. For a review of buffering in insect blood see Buck (1953, pp. 182–186) and Levenbook (1950).

CO_2 capacity and pH to ambient CO_2 concentration (Punt, Parser and Kuchlein; Buck and Friedman) and by the relative smoothness of the buffer capacity curves for insect blood in general (*e.g., Prodenia,* Babers, 1941; and *Gastrophilus,* Levenbook, 1950). There is no indication, in other words, that a *sudden* increase in rate of evolution of CO_2 could occur during the progressive accumulation of lactic acid, CO_2 or other normal acid metabolite. The second general possibility does not appear likely upon any conventional metabolic or biochemical grounds, nor has any evidence for its occurrence been adduced. In *Agapema* and *Hyalophora,* in fact, the loss, during the burst, of the amount of CO_2 impounded during the entire interburst period should involve at most a change in blood pH of only 0.02 units (Buck and Friedman).

A hydrogen ion trigger for the burst is also contraindicated on the grounds that it would relegate spiracular activity to incidental or at least secondary significance in the cycle, in defiance of all the evidence of its primary involvement (Buck and Keister, 1955; Schneiderman, 1956; Buck, 1957). In particular, the fact that a burst can be induced by hypoxia or by mechanical intubation of a valve, treatments which are alike in involving the spiracles but are unrelated biochemically to each other (and also, at least in the case of intubation, unrelated to sudden generation of hydrogen ions), strongly favors spiracular dilation as the primary trigger of the CO_2 burst. In this connection it is significant that the triggering of a burst by hypoxia or by intubation can occur *at any stage of the cycle,* even just after a normal burst has concluded, and that the evolution of CO_2 does not cease, as normally, after a given volume has escaped, but, if the valves are kept open, continues until all the reserve CO_2 in the body has escaped (Buck and Keister, 1955). It is significant also that in the only work in which the effects of CO_2 and hydrogen ion on spiracles have been tested independently (Case, 1957), dilation was shown to be controlled by CO_2 tension, not by acidity. Finally, spiracular valve movement depends on muscles and since muscles typically give sudden responses, once a threshold level of stimulating agent is reached, and show ready reversibility of action, it might be argued that spiracular triggering of the burst has more intrinsic probability than a sudden break in a buffer system.

If a direct chemical triggering of CO_2 evolution is rejected, it remains to propose a mechanism by which the physical act of spiracular dilation could cause unloading of bound CO_2. It appears that this would come about rather naturally by mass action effect. The connecting of the tracheal gas space with the CO_2-free ambient atmosphere should cause the depletion of both the CO_2 in the gas phase and that in the aqueous (blood and tissue) phase with which it was effectively in equilibrium during the interburst period. This depletion should promote the dissociation of the small amount of H_2CO_3 present and this in turn should induce the formation of more H_2CO_3 from bicarbonate. The bicarbonate may be converted to carbonic acid through exchange reactions with proteins, which are the main blood buffers in many insects, or with certain free amino acids.

In sum, the observed time courses of both O_2 and CO_2 exchange appear to be satisfactorily accounted for without invoking hypoxia. Punt's diaferometric records therefore in general provide an excellent fit to the predictions of the flow-diffusion theory. The only considerable discrepancy concerns the gradual rise in CO_2 release rate during the interburst period which should occur if CO_2 is being impounded (unless, as seems highly unlikely, the valve tightens progressively so as to keep leak rate constant). Such a rise does not appear to be present in the diaferometer records, although it is difficult to be certain because of prevalence of galvanometer drift and absence of reference baseline. In *Agapema,* such a rise was demonstrated statistically even though individual Warburg records did not appear to show it (Buck and Keister, 1958), and it is conceivable that the same would be true of the diaferometer measurements.

VII. Discussion and Summary

In certain non-ventilating insects the metabolic CO_2 is impounded for long periods alternating with brief, sudden "bursts" of rapid release. During the burst the spiracular valves are open wide, and during the interburst period they are constricted. The starting point of this essay is the fact that the rate of CO_2 output during the interburst period is so low, in comparison with the concurrent rate of O_2 uptake, that it cannot be explained on the basis of pure diffusion. The thesis is put forward that the interburst valves are sufficiently restricted that (a) O_2 cannot at first diffuse inward as fast as it is being consumed, hence (b) a slight negative intratracheal pressure develops which in turn (c) induces a mass in-flow of air that (d) both augments the inward transfer of O_2 and impedes the out-diffusion of CO_2. In support of this thesis:

1. It is shown by computations based on measured valve length and tracheal pCO_2 that rates of air in-flow can exist that account not only for the degrees of interburst CO_2 retention actually observed in pupae of the saturniid moths *Agapema* and *Hyalophora* (cecropia), but for a wide range of theoretical O_2/CO_2 transfer ratios.
2. The computations provide not only for a steady-state of disparate O_2/CO_2 transfer but for a concurrent equilibrium between the in-flow of N_2 of the air and the out-diffusion of N_2 which accumulated in the trachea during the initial stages of air in-flow.
3. The values of tracheal pO_2, tracheal pN_2, interburst spiracular valve area and total trans-spiracular pressure head which are necessary to satisfy the requirements of an interburst flow-diffusion steady-state are physiologically and anatomically reasonable.
4. The theory is compatible with changes in pupal CO_2 release rate which occur in response to changing ambient pO_2, temperature and metabolic rate.
5. The theory is compatible with the observed time courses of both O_2 and CO_2 transfer during both the burst and interburst periods of the cycle.
6. Although not critically affecting the flow-diffusion theory, it is of general interest that empirical and theoretical considerations indicate that (a) the spiracular valves are the primary trigger of the burst, (b) the area of the constricted interburst valve is modulated with respect to ambient pO_2, (c) valve dilation at the burst is a response to intratracheal pCO_2, not tissue pH, and (d) hypoxia does not occur during the cycle.

It should be emphasized that the principal objective of this paper is to demonstrate the theoretical existence of an internally-consistent quasi-steady-state of gas exchange in which O_2 may pass into the respiratory system at many times the rate at which CO_2 escapes. The actual magnitudes of the valve area, tracheal pO_2, pN_2 and total pressure head which are required to fit a given set of empirical data are of quite secondary significance, and they are at least slightly in error because of various simplifying assumptions and computational shortcuts. Furthermore, I have not attempted to deal quantitatively with the transition period at the end of the burst, during which the (unknown) intratracheal gas tensions prevailing just before spiracular constriction must change abruptly to those that appear to persist throughout most of the interburst period. However, a sufficient range of absolute respiratory rates, true respiratory quotients, CO_2 retention ratios and spiracular dimensions has been explored (Table III) to give confidence that an arithmetical fit can be made, and biologically reasonable parameters derived, for any set of empirical requirements likely to be found in insects or other organisms in which gas exchange occurs through small apertures.

Although the theoretical basis of flow-diffusion transfer in gases has apparently not been studied in detail, nor has the quantitative description of a flow-diffusion steady-state been attempted previously, there is abundant empirical evidence both for the actual existence of flow-diffusion and of situations in which it should occur. For example, the development of a negative intratracheal pressure due to O_2 being lost faster by diffusion than it can be replaced by other gases has been amply demonstrated (Buck and Keister, 1956). The possibility of simultaneous involvement of flow and diffusion across the alveolar neck of the mammalian lung was suggested by McCutcheon (1953), though on the basis of a defective model experiment, and it has been shown (Volhard, 1908; Draper and Whitehead, 1949; Holmdahl, 1956) that non-ventilating, denitrogenated mammals can be maintained for over an hour purely on O_2 that is aspirated through a tracheal cannula due to diffusive uptake of O_2 in the alveoli. In such animals the transfer path is so long that the in-flow essentially prevents any out-diffusion of CO_2, and there is, of course, no N_2 equilibrium involved. Nevertheless, the preparation clearly demonstrates the suction that can be set up by normal respiration, the slowness of the rise of pCO_2 in blood and tissue buffers, and the effectiveness of counterflow in impounding CO_2. Non-replacement of pulmonary O_2 by CO_2 can also be seen in the findings of Otis, Rahn and Fenn (1948) on gas exchange in man when the breath is held. Here, intra-alveolar respiratory quotients of 0.1–0.2 were found—*i.e.*, CO_2 escape from the tissues did not nearly compensate for O_2 uptake. On the botanical side, Scholander, van Dam and Scholander (1955) have recognized the occurrence of respiration-dependent air in-flow in mangrove roots, actually measured the suction that develops, and inferred that the internal pN_2 must rise because of "the very slight changes in carbon dioxide concentrations as compared to the oxygen variations." Finally, the very recent direct analyses of tracheal gas in cecropia pupae by Levy and Schneiderman (1957), in which interburst O_2 concentration was stable at 4.6% and interburst CO_2 concentration rose gradually from 4.2 to 6.4%, practically prove the existence of an elevated interburst pN_2 and provide rather convincing evidence for the existence of a flow-diffusion mechanism of the sort defined in the present paper. In this connection it is interesting that in almost all published analyses of insect tracheal gas, the combined O_2 and CO_2 concentrations fall well below 21%. This suggests the presence of a higher-than-atmospheric tracheal pN_2, and tends to strengthen the idea that the CO_2 burst cycle may be an exaggeration of a normal respiratory behavior which is unexpectedly widespread in non-ventilating insects with valvular spiracles (Buck, 1957; Buck and Keister, 1958).

In sum, it appears that the simultaneous occurrence of both bulk flow and diffusion in respiration is neither theoretically questionable, insofar as mechanism is concerned, nor particularly novel, insofar as its probable occurrence in a number of widely diverse organisms is concerned. In spite of this, the unique properties and potentialities of the system seem not to have been clearly recognized heretofore, either biologically, in connection with non-metabolic CO_2 retention, and production of spurious respiratory quotients, or physically, as a situation in which, for example, a "permanent" concentration excess of N_2 can be maintained within a space. Perhaps the extraordinary nature of flow-diffusion gas transport, and its descriptive similarity to active uptake in liquid phase systems, are best thrown into relief by the statement that the interburst period is one in which the pupa is, in effect, selectively filtering O_2 molecules out of the ambient air. In the exploration of the properties of this unusual gas-phase system, the principal aims of the present investigation have been (1) to suggest a way in which "the system avoids getting jammed with nitrogen" (Scholander, van Dam and Scholander), (2) to work out some sets of arithmetically compatible respiratory parameters that are both physiologically reasonable and capable of accounting for a wide range of degrees of CO_2 retention, and (3) to call attention to the capabilities of minute total pressure heads in maintaining physiologically significant bulk gas flows.

The list of colleagues to whom I am deeply in debt for encouragement and help is a measure of my misgivings about being forced to venture into a relatively uncharted domain. Drs. Rubert Anderson, Heinz Specht and Jacob Verduin in particular have been active as basic educators throughout the evolution of the theory, and some of their specific contributions have been acknowledged elsewhere. In addition the study has benefited greatly, on both the physical and biochemical sides, from my discussions with Drs. F. S. Brackett, John Hearon, E. H. Kennard, L. Levenbook, Robert Ramsey, John Severinghaus and John Weske. Rather early in the game, Dr. F. A. Brown, Jr. urged on me the idea of flow-retention, as seen in his pure O_2 respirometer (Brown, 1954), but I was for a long time doubtful of its involvement because I could not see how progressive accumulation of N_2 could be avoided during respiration in air. Finally, however, Dr. Roscoe Bartlett suggested that the intractable problem of gas transfer in a ternary system might yield to successive approximations, and this proposal led eventually to the present method of dealing with the N_2 equilibrium quantitatively.

LITERATURE CITED

Babers, Frank H., 1941. The buffer capacity of the blood of the sixth-instar southern armyworm (*Prodenia eridania*). *J. Agric. Res.*, **63**: 183–190.

Bishop, George H., 1923. Body fluid of the honeybee larva. I. Osmotic pressure, specific gravity, pH, O_2 capacity, CO_2 capacity, and buffer value, and their changes with larval activity in metamorphosis. *J. Biol. Chem.*, **58**: 543–565.

Brown, F. A., Jr., 1954. A simple, automatic, continuous-recording respirometer. *Rev. Sci. Instr.*, **25**: 415–417.

Buck, John, 1953. Physical properties and chemical composition of insect blood. Pp. 147–190 in "Insect Physiology," ed. by Kenneth D. Roeder, Wiley, New York.

Buck, John, 1957. Triggering of insect spiracular valves. Pp. 72–79 in "Physiological Triggers, etc.," ed. by T. H. Bullock. Amer. Physiol. Soc., Washington.

Buck, John, and Stanley Friedman, 1958. Cyclic CO_2 release in diapausing pupae. III. CO_2 capacity of the blood: Carbonic anhydrase. *J. Insect Physiol.*, 2: (1).

Buck, John, and Margaret Keister, 1955. Cyclic CO_2 release in diapausing *Agapema* pupae. *Biol. Bull.*, **109**: 144–163.

Buck, John, and Margaret Keister, 1956. Diffusion phenomena in the tracheae of *Phormia* larvae. *Physiol. Zoöl.*, **29**: 137–146.

Buck, John, and Margaret Keister, 1958. Cyclic CO_2 release in diapausing pupae. II. Tracheal anatomy, volume and pCO_2; blood volume; interburst CO_2 release rate. *J. Insect Physiol.*, **1**: 327–340.

Buck, John, Margaret Keister and H. Specht, 1953. Discontinuous respiration in diapausing *Agapema* pupae. *Anat. Rec.*, **117**: 541.

Case, James F., 1956. Carbon dioxide and oxygen effects on the spiracles of flies. *Physiol. Zoöl.*, **29**: 163–171.

Case, James F., 1957. Differentiation of the effects of pH and CO_2 on spiracular function of insects. *J. Cell. Comp. Physiol.*, **49**: 103–114.

Crank, J., 1956. The mathematics of diffusion. Oxford.

Draper, William B., and Richard W. Whitehead, 1949. The phenomenon of diffusion respiration. *Current Res. in Anesthesia and Analgesia*, **28**: 307–318.

Garby, Lars, 1957. Studies on transfer of matter across membranes with special reference to the isolated human amniotic membrane and the exchange of amniotic fluid. *Acta Physiol. Scand.*, **40**. Suppl. 137. 84 pp.

Hazelhoff, E. H., 1926. On a new form of breathing regulation (regulation of diffusion) in insects and arachnida. *Proc. Sect. Sci. Koninkl. Akad. Wetensch. Amsterdam*, **29**: 492–496.

Heller, Josef, 1930. Sauerstoffverbrauch der Schmetterlingspuppen in Abhängigkeit von der Temperatur. *Zeitschr. vergl. Physiol.*, **11**: 448–460.

Holmdahl, Martin H., 1956. Pulmonary uptake of oxygen, acid-base metabolism, and circulation during prolonged apnoea. *Acta Chirurg. Scand.* Suppl. 212. 128 pp.

Ito, Toshio, 1954. Discontinuous output of carbon dioxide by undifferentiated *Bombyx* pupae. *Jap. J. Appl. Zool.*, **19**: 98.

Jacobs, M. H., 1935. Diffusion processes. *Ergeb. der Biol.*, **12**: 1–160.

Jost, W., 1952. Diffusion in solids, liquids, gases. Academic Press, N. Y.

Koefoed-Johnson, Valborg, and Hans H. Ussing, 1953. The contributions of diffusion and flow to the passage of D_2O through living membranes. *Acta Physiol. Scand.*, **28**: 60–76.

Levenbook, L., 1950. The physiology of carbon dioxide transport in insect blood. I. The form of carbon dioxide present in Gastrophilus larva blood. *J. Exp. Biol.*, **27**: 158–174; Ibid. III. The buffer capacity of Gastrophilus blood. *J. Exp. Biol.*, **27**: 184–191.

Levy, Robert I., and Howard A. Schneiderman, 1957. The direct measurement and significance of changes in intratracheal gas composition during the respiratory cycle of the cecropia moth. *Anat. Rec.*, **128**: 583.

McCutcheon, F. H., 1953. Atmospheric respiration and the complex cycles in mammalian breathing mechanisms. *J. Cell. Comp. Physiol.*, **41**: 291–303.

Otis, A. B., H. Rahn and W. O. Fenn, 1948. Alveolar gas changes during breath holding. *Amer. J. Physiol.*, **152**: 674-686.

Punt, A., 1944. De gaswisseling van enkele bloedzuigende parasieten van warmbloedige dieren (*Cimex, Rhodnius, Triatoma*). *Onderz. Physiol. Lab. Rijks-Univ. Utrecht, 8th Ser.*, **3**: 122–141.

Punt, A., 1950. The respiration of insects. *Physiol. Comp. Oecol.*, **2**: 59–74.

Punt, A., 1956. The influence of carbon dioxide on the respiration of *Carabus nemoralis* Müll. *Physiol. Comp. Oecol.*, **4**: 132–141.

Punt, A., W. J. Parser and J. Kuchlein, 1957. Oxygen uptake in insects with cyclic CO_2 release. *Biol. Bull.*, **112**: 108–119.

SCHNEIDERMAN, HOWARD A., 1956. Spiracular control of discontinuous respiration in insects. *Nature,* **177**: 1169–1171.

SCHNEIDERMAN, HOWARD A., AND CARROLL M. WILLIAMS, 1955. An experimental analysis of the discontinuous respiration of the cecropia silkworm. *Biol. Bull.,* **109**: 123–143.

SCHOLANDER, P. F., L. VAN DAM AND SUSAN I. SCHOLANDER, 1955. Gas exchange in the roots of mangroves. *Amer. J. Bot.,* **42**: 92–98.

VOLHARD, F., 1908. Über künstliche Atmung durch Ventilation der Trachea und eine einfache Vorrichtung zur rhytmischen künstlichen Atmung. *München. med. Wochenschr.,* **55**: 209–211.

WIGGLESWORTH, V. B., 1953. Surface forces in the tracheal system of insects. *Quart. J. Micros. Sci.,* **94**: 507–522.

ZEUTHEN, ERIK, 1955. Comparative physiology (respiration). *Ann. Rev. Physiol.,* **17**: 459–482.

A NEW STOMATOPOD CRUSTACEAN OF THE GENUS LYSIOSQUILLA FROM CAPE COD, MASSACHUSETTS

FENNER A. CHACE, JR.

Smithsonian Institution, Washington, D. C.

Of the eight recognized genera of Recent stomatopods, *Lysiosquilla* is probably the most heterogeneous and most difficult to define satisfactorily. To this genus have been assigned those species that have the carapace devoid of carinae and without a complete cervical groove; the abdomen flattened dorsally and without longitudinal carinae on the first five somites; the telson without a distinct median carina; the antepenultimate segment of the raptorial claw grooved for its entire length and not produced proximally beyond its articulation with the preceding segment; the penultimate segment of the claw finely pectinate or spinose along the outer part of its dorsal edge; and the terminal segment armed with four or more teeth and not inflated proximally. The nearly 40 known species and subspecies of *Lysiosquilla* include the largest and some of the smallest stomatopods. Only seven of them have been previously recorded with certainty from the western Atlantic, and none have been added to the genus from this region since 1900. *L. scolopendra* (Latreille, 1825) must be treated as a doubtful species, possibly synonymous with *L. excavatrix* Brooks, 1886, and *L. plumata* and *L. maiaguesensis,* both described from immature specimens by Bigelow in 1901, were correctly transferred to *Pseudosquilla* by Schmitt (1940). The larval forms collected by the "Challenger" Expedition off St. Vincent, British West Indies, and variously called *Coronis* (*Erichthus*) *minutus, Lysiosquilla* (*Coronis*) *minutus, Erichthus* (*Coronis*) *minutus,* and *Lysiosquilla* (*Lysioerichthus*) *minutus* by Brooks (1886) cannot be identified with the adults of any known species until the life-histories of those from the western Atlantic are known much better than they are at present.

As material of all of the species previously described from the American Atlantic was available for study, a key to these species has been appended to this paper. The publications by Balss, Bigelow, Kemp, Lemos de Castro, and Schmitt, listed in the bibliography, are the most important ones for students of American stomatopods; from them, references to most of the scattered literature on the group can be obtained.

I am very grateful to Mr. Milton B. Gray of the Marine Biological Laboratory, Woods Hole, Massachusetts, for collecting the material on which the following description is based and to Dr. Marian H. Pettibone of the University of New Hampshire for calling my attention to the species.

Lysiosquilla grayi, n. sp.

Material examined: Bass River, Yarmouth, Massachusetts; burrowing in muddy sand near low-water mark; Milton B. Gray, collector; May 1, 1954; 2 males. Same;

July 31, 1954; 2 females (one is holotype, U. S. National Museum Cat. No. 100931). Same; May 23, 1955; 4 males, 4 females. Same; March 18, 1957; 2 males, 5 females.

Description: Carapace very short, about as long as combined lengths of first three exposed thoracic somites; smooth dorsally, with vestige of cervical groove on inner part of each lateral plate; lateral angles broadly rounded. Rostral plate smooth, subrectangular with convex lateral margins, distinctly broader than long, and terminating anteriorly in an obtuse angle. First exposed thoracic somite unarmed but with rounded anterolateral lobe that gives shallowly bilobed appearance to lateral margins. Lateral margins of other three exposed thoracic somites slightly concave in lateral view. Abdominal somites smooth and unarmed except for sharp posterolateral angles of sixth somite. Telson about half again as wide as long, smooth, swollen, and unarmed dorsally except for three very broadly obtuse lobes which form a nearly entire marginal eave overhanging true posterior margin. Marginal armature consisting of five pairs of sharp posterolateral fixed teeth, a row of 15 to 18 slender, upcurved, fixed posterior spines, and a larger, movable, upcurved, ventral spine at each end of row of posterior spines.

Cornea broad, set obliquely on stalk, and bulging outward laterally but not bilobed. Antennular peduncle exceeding eye by most of terminal segment. Antennal peduncle slightly exceeding antennal scale but not reaching beyond middle of cornea. Mandibular palp lacking. Antepenultimate segment of raptorial claw not carinate but with acute tooth at distal end of dorsal margin. Penultimate segment with four movable spines on inner side below pectinate margin, first folded into groove and third much the smallest. Terminal segment armed with 11 to 15 teeth, including apical one; opposite margin with prominent blunt lobe at base, followed by a low, broadly obtuse one. First two pairs of exposed thoracic appendages with shorter branch very broadly oval, almost subcircular; that of third pair more narrowly ovate. Basal segment of uropod with two long spines, inner one stouter, longer, and more prominently carinate than outer one; basal segment of outer branch with six movable, spatulate spines, distal one strongly curved and reaching to middle of terminal segment of outer branch.

Size: Holotype female approximately 40 mm. long from tip of rostral plate to end of telson; carapace, not including rostral plate, 5.5 mm. long. There is little size variation in the specimens examined: males have a carapace length of 5.0 to 6.0 mm., and females of 5.3 to 6.0 mm.

Color: Scattered dark chromatophores on a creamy surface in both sexes. In the figured holotype, the chromatophores are more numerous than usual because the specimen was apparently approaching a molt and chromatophores are visible in the underlying cuticle.

Remarks: Lysiosquilla grayi is very closely related to *L. decemspinosa* Rathbun, 1910, which occurs in a similar habitat in Peru, and Costa Rica. All of the seven known specimens of the latter species are smaller than those of *L. grayi*, the carapace lengths ranging from 2.5 to 3.3 mm. Schmitt (1940) believed that all of these specimens might be immature. All but one of the specimens differ from the Atlantic form in having only three pairs of fixed posterolateral teeth on the telson, but one of them, a male with a carapace length of 2.7 mm., has two additional pairs of small teeth, making a total of five pairs as in *L. grayi*. Perhaps of more significance is the fact that the number of fixed posterior spines on the telson ranges from 18 to 23 in *L. decemspinosa,* whereas there are only 15 to 18 in *L. grayi.* Also, there are 10 to 12 teeth on the terminal segment of the raptorial claw in the Pacific species, as contrasted with 11 to 15 in the Atlantic one. Until additional material becomes available, it seems best to consider the present species distinct from its Pacific analogue.

The possibility that *L. grayi* is the adult stage of *L. minuta* Brooks, 1886, from the Caribbean region cannot be disregarded entirely. As mentioned above, however, this larval form must remain a questionable species until we know more of the life-histories of the American stomatopods.

Both *L. grayi* and *L. decemspinosa* are unusual in lacking a mandibular palp. Most of the known species of *Lysiosquilla,* including all of those previously described from the western Atlantic, have a three-segmented mandibular palp, but this character cannot be considered of more than specific importance until the entire genus is reviewed. As pointed out by Kemp (1913), there are several stomatopod genera in which the mandibular palp may be either well developed or entirely absent.

The color pattern in both sexes of *L. grayi* is very similar to that in the female of *L. excavata* Brooks, but males of the latter species are uniformly dark brown.

The western Atlantic species of *Lysiosquilla,* although few in number, demonstrate the possible evolution of the telson in this genus remarkably well. In *L. glabriuscula,* the dorsal surface of the telson bears a slightly raised, triangular, median boss, the apex of which does not nearly reach the posterior margin. In *L. scabricauda,* there is a similar triangular boss, but it is more elevated, especially at the apex which, however, does not reach to the posterior margin. In *L. polydactyla,* there is no raised area, but a flattened, faintly bilobed median lobe, which may represent the apex of the triangular area in the preceding species, projects above and beyond the posterior margin of the telson, and a sharp tooth projects posteriorly from the dorsal surface on either side of the median lobe. A similar arrangement is found in *L. platensis,* but the median lobe is faintly carinate in the midline and distally trilobate, and the lateral teeth are acute lobes supported by prominent carinae. There is a similar, flattened, distally trilobate projecting lobe in *L. armata* but it is flanked by two sharp spines on either side. In *L. biminiensis,* the median projection is a sharp spine which is flanked on each side by two similar spines on a slightly lower level. In *L. grayi,* the median and submedian projections are so broadly obtuse and poorly defined as to form a nearly entire false margin projecting over the true posterior margin. Possibly the most advanced stage is represented by *L. excavata,* in which the posterior projections are also broadly obtuse and ill defined, but the false margin thus formed is so little separated from the true posterior margin that the latter practically disappears, and the marginal spines seem to arise from the ventral surface of the telson.

Key to the Western Atlantic Species of Lysiosquilla

1. No submarginal spines on ventral surface of telson....................................2
   One or more pairs of submarginal spines apparently arising from ventral surface of telson..6
2. Triangular median area on dorsal surface of telson not distinctly projecting posteriorly. Basal segment of uropod with two spines. Shorter branch of all exposed thoracic legs straplike, curved....................................3

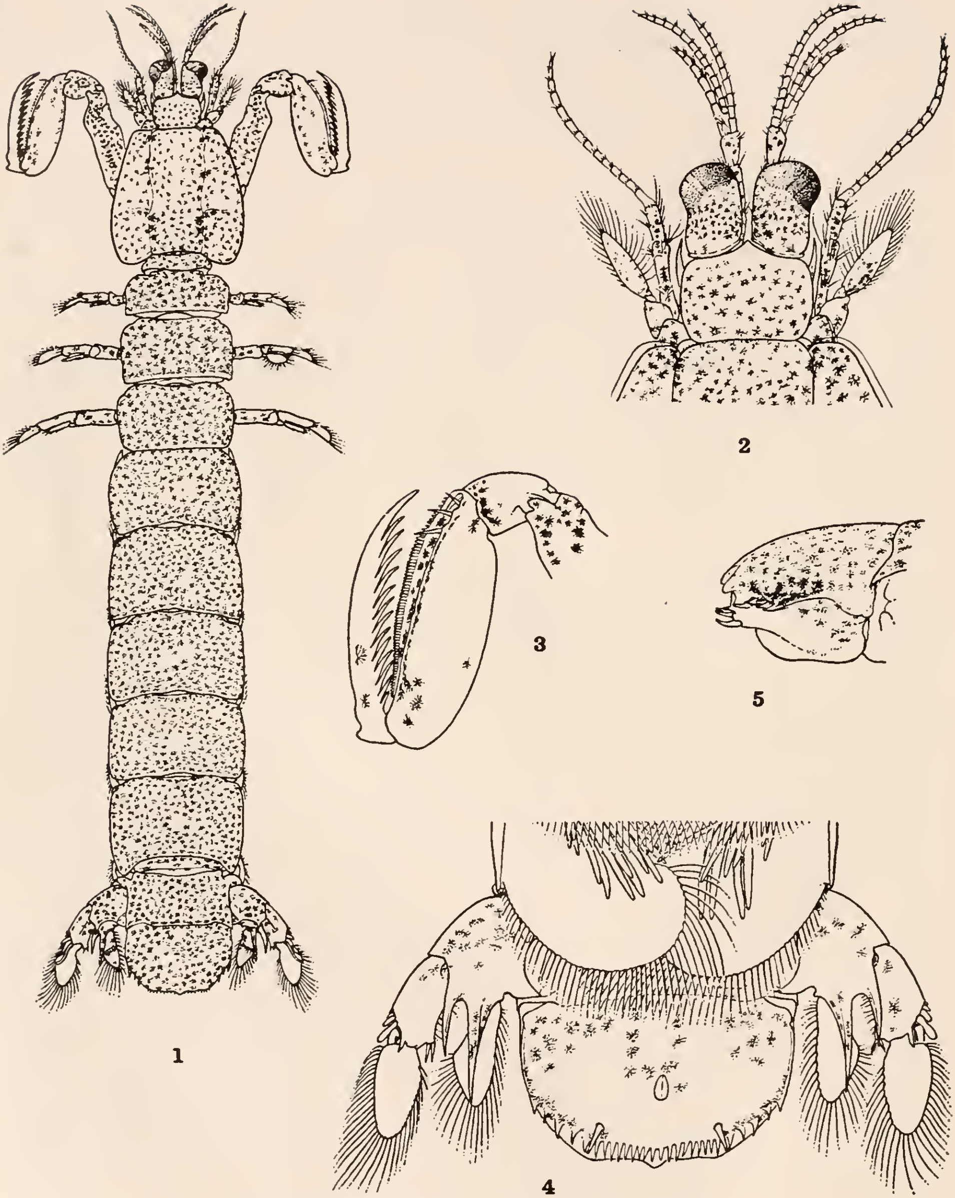


Plate I. *Lysiosquilla grayi,* female holotype.

Figure 1. Dorsal view of entire animal. × 3.
Figure 2. Dorsal view of anterior end. × 7.5.
Figure 3. Inner surface of right raptorial claw. × 7.5.
Figure 4. Ventral surface of posterior end. × 7.5.
Figure 5. Lateral view of telson. × 7.5.

Telson with a median, dorsal, tonguelike projection reaching to or beyond posterior margin. Basal segment of uropod with three spines. Shorter branch of all exposed thoracic legs ovate..4

3. Abdomen unarmed. Dactyl of raptorial claw with 5 or 6 teeth, including terminal one ....*L. glabriuscula* (Lamarck, 1818)

Fifth and sixth abdominal somites with posterior margins spinose in adult specimens. Dactyl of raptorial claw with 8 to 12 teeth......................*L. scabricauda* (Lamarck, 1818)

4. Fourth and fifth abdominal somites unarmed. Basal segment of uropod with outer, distal spine longer than penultimate one...5

Fourth, fifth, and sixth abdominal somites spinose posteriorly. Basal segment of uropod with outer, distal spine shorter than penultimate. Dactyl of raptorial claw with 10 or 11 teeth, including terminal one...................................*L. armata* Smith, 1881

5. Rostral plate wider than long. Dorsal surface of telson with pair of sharp submedian carinae terminating posteriorly in strong submarginal teeth. Dactyl of raptorial claw with 13 or 14 teeth, including terminal one...................................*L. platensis* Berg, 1900

Rostral plate longer than wide. Telson with pair of submedian dorsal projections but no sharp carinae. Dactyl of raptorial claw with 17 to 20 teeth.......................... ....*L. polydactyla* Von Martens, 1881

6. Rostral plate subrectangular or subtrapezoidal. First segment of exposed thoracic legs unarmed. Basal segment of uropod with inner spine about half again as long as outer one..7

Rostral plate heart-shaped. First segment of exposed thoracic legs with posterolateral spine. Basal segment of uropod with outer spine very broad and about twice as long as slender inner one. Dactyl of raptorial claw with 15 or 16 teeth..*L. excavatrix* Brooks, 1886

7. Rostral plate narrowing distally, about as broad as long. Telson with row of five strong dorsal spines extending to, or slightly beyond, posterior margin; movable, submedian spines arising below margin. Dactyl of raptorial claw with 15 or 16 teeth..................... ....*L. biminiensis* Bigelow, 1893

Rostral plate subrectangular, much broader than long. Dorsal surface of telson unarmed but produced posteriorly in an eave or false margin overhanging marginal spines. Dactyl of raptorial claw with 11 to 15 teeth................................*L. grayi* (see above)

LITERATURE CITED

Balss, H., 1938. Stomatopoda, in H. G. Bronn's Klassen und Ordnungen des Tierreichs, 5 (Abt. 1, Buch 6, Teil 2) : 1–173.

Bigelow, R. P., 1894. Report on the Crustacea of the order Stomatopoda collected by the Steamer Albatross between 1885 and 1891, and on other specimens in the U. S. National Museum. *Proc. U. S. Nat. Mus.*, **17**: 489–550.

Bigelow, R. P., 1901. The Stomatopoda of Porto Rico. *Bull. U. S. Fish Comm.*, **20** (2) : 149–160.

Brooks, W. K., 1886. Report on the Stomatopoda collected by H. M. S. Challenger during the years 1873–76. *Rep. Sci. Res. Voy. H. M. S. Challenger, Zool.*, **16** (45) : 1–116.

Kemp, S., 1913. An account of the Crustacea Stomatopoda of the Indo-Pacific Region based on the collection in the Indian Museum. *Mem. Indian Mus.*, **4**: 1–217.

Lemos de Castro, A., 1955. Contribuição ao conhecimento dos crustáceos da ordem *Stomatopoda* do litoral Brasileiro: (Crustacea, Hoplocarida). *Boletim do Museu Nacional, Nova Serie, Rio de Janeiro, Zool.*, No. **128**: 1–68.

Schmitt, W. L., 1940. The stomatopods of the west coast of America based on collections made by the Allan Hancock Expeditions, 1933–1938. *Allan Hancock Pacific Expeditions,* **5**: 129–225.

LIFE–HISTORY AND BIOLOGY OF THE OYSTER CRAB, PINNOTHERES OSTREUM SAY

AAGE MØLLER CHRISTENSEN[1] AND JOHN J. McDERMOTT

Oyster Research Laboratory, Rutgers University, N. J. Agricultural Experiment Station, Bivalve, N. J.

While the adult female of several species of the pea crab, *Pinnotheres,* has been known since ancient times, it is not clear when the first male was observed and described. The earliest reference available to the present authors was found in a paper by Thompson (1835).

He describes the male of *P. pisum* as being firm in texture, with compressed, hairy appendages and of flatter form and much smaller size than the (adult) globular, soft-shelled female. Such hard-shelled *P. pisum* were generally all thought to be males until Orton (1921) demonstrated the existence of hard-shelled females, which except for differences in the genital apertures and the pleopods proved to be indistinguishable from the males. However, hard-shelled females were known in at least four other species of *Pinnotheres* prior to 1921 (Rathbun, 1918). Possibly, Thompson (1835) was also aware of this in *P. pisum* as he states, "For a considerable time the young females are scarcely to be distinguished from the males, and in this stage both differ so much from the adult, as to render it probable that they have often been taken for individuals of different species, . . ."

Orton (1921) was the first to find a soft-shelled male, which except for the same characteristics as mentioned above resembled the immature female of similar size.

A few years later, Atkins (1926) studied and described all the growth stages of *P. pisum* found in *Mytilus edulis* in English waters. As Orton, she regarded the hard-shelled crabs as free-living, invasive crabs, a point of view which the author later abandoned (Atkins, 1954, 1955). Hence the hard-shelled stage of both sexes was designated as Stage I. In the female, four more stages were described, the fifth and last stage being the mature crab. In the male, only the hard-shelled stage was described, no reference being made to Orton's discovery of a soft-shelled specimen. It was stated, however, that a few abnormal males were found. Soft-shelled males were found also by Mercier and Poisson (1929), who stated that they were abnormal due to the influence of an entoniscid parasite. Later Atkins (1933) disproved this statement and expressed the hope of discussing the matter in a later paper as she still considers these males as abnormal.

Stauber (1945) found and described similar growth stages in *P. ostreum* from the American oyster, *Crassostrea virginica.* He therefore followed Atkins (1926) in designating the hard-shelled stage as the first (invasive) stage, which in the female is succeeded by four more stages as in *P. pisum.* Stauber also found a number of soft-shelled males, evidently corresponding with the finds of *P. pisum* mentioned above. With some reservation, he referred these males to a second stage following the hard-shelled stage. This hypothesis does not agree with the general belief that the hard-shelled male is the adult stage of this sex.

---

[1] Present address: Det marinbiologiske laboratorium, Helsingør, Denmark.

Although numerous species of *Pinnotheres* have been described (Bürger, 1895; Rathbun, 1918; Tesch, 1918; and others), knowledge about post-larval stages, other than the adult, is scarce for all species except the two mentioned above. Only in two species has the first crab stage been described, *viz.* in *P. taylori* by Hart (1935), and in *P. ostreum* by Sandoz and Hopkins (1947). In both cases the crabs were reared from the egg in the laboratory, and the stage has never been reported from nature.

The latter paper included a description of the early developmental stages of which there proved to be four zoeal stages, of which the first two had been described earlier (Hyman, 1924), and one megalopa. It therefore almost completed our knowledge of the whole developmental cycle in any pea crab for the first time. The authors, however, pointed out that two or more instars were still unknown as the two crabs reared by them measured only about 0.6 mm. in carapace width while the smallest hard-shelled *P. ostreum* found by Stauber (1945) measured 1.4 mm.

Much to our surprise, the missing instars as well as the first crab stage were found in a number of oyster spat collected in Delaware Bay on August 17th in 1955. This meant that the hard-shelled stage could not be the first invasive stage. The fact that hard-stage crabs of several species of *Pinnotheres* have been taken free in the water (Verrill and Smith,[2] 1874; Rathbun, 1918; Berner, 1952; and others), or trapped between the valves of their host (Orton, 1921; and others), had to be explained otherwise. Two hard-stage oyster crabs were also caught outside their host by the present authors. In addition to the description of the new growth stages, a re-investigation of the biology of *P. ostreum* was therefore decided upon. This seemed especially worth while since Stauber's paper is the only comprehensive work on the biology of any pinnotherid crab. This seems strange considering that the genus *Pinnotheres* alone comprises more than a hundred species, unless, which is very possible, many of them are synonyms. The results of our subsequent studies are the subject of the present paper.

We wish to express our sincere appreciation to Mrs. Grete Møller Christensen, Mr. Donald E. Kunkle and Mr. William Richards for their unfailing interest in our work and for invaluable help in collecting and opening numerous oysters, as well as for help rendered in various other ways. We are much indebted to Dr. Leslie A. Stauber of Rutgers University for reviewing the manuscript, and for giving us access to his collections of oyster crabs as well as his unpublished data on the subject. The director of the N. J. Oyster Research Laboratory, Dr. Harold H. Haskin, gave our work his enthusiastic support for which we express our sincere gratitude. The senior author gratefully acknowledges the grants from the Fulbright Foundation and the Danish State Scientific Foundation which made his visit to the United States possible.

MATERIALS AND METHODS

The present work on the biology and life-history of *P. ostreum* was carried out at the New Jersey Oyster Research Laboratory, Rutgers University, from August, 1955 to December, 1956.

---

[2] Judging by their figure, Pl. I, Fig. 2, the species found was *P. maculatus,* and not *P. ostreum* as stated.

Studies on the rate of growth and development, one of the primary objects, were based on extensive collections in the field. Since such factors as the age and size composition of the host populations, as well as the environmental conditions, could be expected to vary from one area to another, it was important to eliminate as many of these variables as possible. We decided, therefore, to find one or two small grounds in Delaware Bay with a good set of 1955 oyster spat and with a high incidence of infestation with oyster crabs. These grounds were then to be sampled at regular intervals throughout the period of investigation. This procedure enabled us to deal with local populations of oysters and crabs of known year-classes. It also eliminated the risk of dealing with oyster populations exhibiting different incidences of infestation, a factor which later proved to be very important to the interpretation of the assembled data.

One ground was selected at Pierces Point about ten miles north of Cape May Point, and another was selected about two miles west of Pierces Point on the Bay Shore Channel Bed, an area where commercial oyster dredging is prohibited. At Pierces Point, oysters were collected by hand at low water when the oysters were exposed. Here a heavy mortality of the 1955 spat occurred late in the winter of 1955–1956, wherefore sampling was discontinued except for a few samples during the summer of 1956, and sampling of 1956 spat in the fall of that year. On the Bay Shore Channel Bed the depth at mean low water is about 6 meters, and here oysters were obtained from the research vessel "Julius Nelson." Little mortality of the 1955 spat was noted on this ground, but some mortality of the crabs occurred in February and early March of 1956 (Fig. 5).

In addition to the regular collections of crabs from 1955 spat (Table I), other collections, which included crabs from older oysters as well as from 1956 spat, were made on the above mentioned as well as other grounds in Delaware Bay. An effort was made to secure a high number of crabs at each collection. As seen in Table I, the lowest number taken in the series of regular samples was 55, and most of the samples contained more than a hundred crabs. Collections began at a time when most of the 1955 crabs were still in the first crab stage, thus enabling us to study the whole post-planktonic life cycle of the crab.

On the Bay Shore Channel Bed, which constituted our main sampling ground, bottom temperatures were determined with a reversing thermometer on each collecting date.

Oysters brought back to the laboratory were, with few exceptions, examined alive and always under a dissecting microscope. Infested oysters, and from time to time also all of the uninfested oysters collected along with them, were measured to the nearest 0.5 mm. in length with vernier calipers. The crabs were measured under the microscope to the nearest 0.1 mm. in carapace width. The smaller crabs were measured with a calibrated ocular micrometer, while the majority were measured on a millimeter glass ruler or by vernier calipers. The amount of error was judged to be the same for the last two methods, as they were checked on several occasions. Unless otherwise indicated, all crab sizes in the present paper refer to the width of the carapace.

Notes on the general condition, amount of gill damage caused by the crab, and other pertinent data concerning the infested oysters were taken on the majority of the collections.

All of the oyster crabs found, except those used for dissections and experiments, were preserved in alcohol, and specimens of the new instars have been deposited in the United States National Museum and in the Zoology Museum of the University of Copenhagen, Denmark.

For various reasons it was decided to reserve a detailed description of the new instars and the necessary revision of the numbering of all the post-planktonic growth stages to a second paper. Consequently the present paper only includes such brief notes on the new instars, as well as the previously described stages, as is necessary to the understanding of the following account of the life-history and biology of the crab.

TABLE I

Number and mean size for each collection of oysters and oyster crabs of the 1955 year-class taken at Pierces Point and the Bay Shore Channel Bed, with a column showing the incidence of infestation

Date of collection	Number of infested oysters	Mean length of oysters in mm.	Total number of *Pinnotheres*	Range in carapace width in mm.	Mean carapace width in mm.	Incidence of infestation in per cent
			Pierces Point			
17– 9–55	167	13.8	279	0.6– 2.4	0.69	69.0
1–11–55	101	15.4	107	0.7– 2.1	1.35	60.1
5–12–55	175	18.1	185	0.7– 2.2	1.42	56.6
13–12–55	—	—	215	0.6– 2.0	1.31	—
4– 1–56	127	19.2	130	0.7– 2.2	1.46	64.8
25– 1–56	55	17.4	55	0.8– 2.1	1.51	56.1
23– 2–56	98	18.9	99	0.9– 2.5	1.52	56.4
22– 3–56	85	19.2	86	0.8– 2.2	1.50	43.6
7– 7–56	16	30.6	16	2.5– 4.7	3.10	11.7
			Bay Shore Channel Bed			
14–12–55	192	—	193	0.6– 3.1	1.54	68.3
6– 1–56	120	19.1	130	0.7– 2.6	1.47	72.8
4– 2–56	134	21.7	139	0.7– 2.7	1.59	72.4
5– 3–56	81	22.5	81	1.0– 2.8	1.75	55.1
18– 4–56	127	22.0	127	0.7– 3.2	1.79	52.9
3– 5–56	124	21.1	124	0.9– 2.6	1.63*	59.9
22– 5–56	96	22.6	96	0.9– 2.7	1.75	58.9
5– 6–56	103	24.9	104	0.8– 2.9	1.95	53.1
14– 6–56	110	25.0	110	1.6– 4.2	2.35	62.5
20– 6–56	100	25.4	100	1.1– 5.8	2.46*	58.1
6– 7–56	116	34.3	124	1.8– 7.8	3.64	41.1
11– 7–56	103	35.7	106	1.8– 7.7	4.03	35.7
18– 7–56	108	39.7	113	2.2– 9.2	5.18	35.0
26– 7–56	118	41.6	119	2.5– 9.6	5.76	33.1
1– 8–56	63	44.8	63	2.8– 8.6	6.24	35.0
16– 8–56	118	43.3	119	2.2– 9.6	6.60	28.9
12– 9–56	114	49.2	114	4.4– 9.8	7.41	33.6
9–10–56	100	51.7	100	5.2–10.0	7.68	34.4

* Measurements from formalin preserved specimens.

Laboratory experiments and observations were carried out to a limited extent, and some of the moulting experiments yielded valuable information. Holes were chipped in the ventral portion of oysters to insert crabs of known stage, sex and size with the hope that moulting might occur. Although only a small percentage of the crabs moulted, the method proved valuable since crabs kept in Petri dishes did not moult at all except in those cases where the crabs were obviously ready to moult on arrival to the laboratory. Oysters for the above purpose were generally collected from the upper parts of the Delaware Bay Natural Seed Beds where the percentage of oysters infested with the oyster crab was very low. Occurrence of moulting inside the oysters could be detected without opening them since the cast exoskeleton is ejected shortly after a moulting has taken place.

Growth Stages in Pinnotheres ostreum

As indicated earlier, the hard-shelled stage is not the first invasive stage. The true invasive stage is the first crab stage. Proof of this are the following facts: 1. The morphology of the first crab stage shows adaptations both for a free-swimming existence and for entering the host. 2. The first crab stage was found abundantly inside oysters but it was also collected in plankton samples in Delaware Bay. 3. While all subsequent stages were found inside oysters, no earlier stages, such as the megalopa which was suggested by Atkins (1954) to be the invasive stage in *P. pinnotheres,* were ever taken in the host animals.

In the following, therefore, Stauber's (1945) Stage I will be referred to as the *hard stage*. The new instars between the *invasive stage* and the hard stage will be called the *pre-hard stages,* a term which is arbitrarily defined as excluding the invasive stage. To avoid confusion with the earlier literature, and because we have not yet been able to assign pre-hard crabs to definite growth stages, we have adhered to the numbering of the *post-hard stages* as given by Stauber, except for the male in which we found that no post-hard stage exists. In addition to the following remarks, the summary of the main characteristics of all post-planktonic growth stages presented in Table II should be helpful to the reader.

Invasive stage

The mean size of the two crabs reared by Sandoz and Hopkins (1947) was 0.59 mm. while the mean of 183 specimens collected by us at Pierces Point on September 17, 1955 was 0.65 mm. with a size range from 0.59 to 0.73 mm.

The invasive stage is similar to the hard stage in many respects, which is remarkable considering that the pre-hard instars separating these two stages in the developmental cycle have a very different morphology. Both of these stages have a flat carapace, flattened pereiopods with thickened posterior borders, and long, plumose swimming hairs on the third and fourth pairs. They also have, in contrast to all other stages, two characteristic whitish spots visible both on the carapace and on the sternum. These spots seem somewhat larger in the invasive stage than figured by Sandoz and Hopkins, who apparently failed to note them on the dorsal side. However, in proportion to the size of the crab they are much smaller in the invasive stage than in the hard stage. These spots mark the ends of two solid, cylindrical rods connecting the dorsal and ventral side of the body. They consist of a very hard, opaque substance and serve as attachments for many muscles, which probably to a large extent are the heavy musculature needed for the quick swimming movements of the third and fourth pairs of pereiopods. When swimming, only these pereiopods are used while the other pairs, especially the fifth, are kept more or less motionless. Although not as soft-shelled as the pre-hard stages, the invasive stage is not nearly as firm as the hard stage.

We found that the males leave their hosts in the hard stage and proceed to enter other oysters for copulatory purposes, and indications are that the female also may change host under certain circumstances. It is, therefore, not to be wondered that the invasive stage has so many structural similarities common with the hard stage and differing from all other stages. They are all adaptive modifications instrumental for a free-living existence as well as for the invasion of the host.

Pre-hard stages

These are the hitherto undescribed instars between the invasive stage and the hard stage.

Morphologically these stages resemble post-hard crabs. Like these they have a rounded, soft-shelled carapace which yields to the touch. The pereiopods are slender and without swimming hairs. More especially they resemble the second stage described by Stauber (1945). In fact we cannot distinguish with certainty between the last pre-hard and the second stage crab. Although this reflects the morphological adaptation of these stages for life within the oyster, it is still remarkable considering that the very distinctive hard stage separates them in the developmental cycle. As the male seldom, if ever, develops beyond the hard stage, this problem of stage identification applies, however, mainly to the female. It is hoped that future comparative studies of a large number of young females may make a true distinction possible.

As the smallest hard-stage crab, a female, found in our large collection measured 1.3 mm., it seems fairly certain that all soft-shelled crabs smaller than this must be pre-hard crabs. The smallest specimen found measured about 0.75 mm., and several moults, probably at least four, occur with increase in body size and development of the pleopods before the crab moults into the hard stage.

The sexes are indistinguishable except for differences in genital openings and morphology and number of pleopods. By a careful microscopical examination of the latter it was possible to determine the sex of all crabs down to a size of about 0.9 mm. This meant that practically all but the first of the pre-hard stages could be sexed with certainty.

Stauber (1945) showed that hard-stage males were larger on the average than hard-stage females, and this is also true for pre-hard crabs. Admittedly the maximum size, and therefore also the mean, of pre-hard females cannot be stated as long as the last of these stages can be confused with the second stage. Nevertheless, it is bound to be considerably smaller in the female than in the male since the largest hard stage female found measured only 2.7 mm. as against 4.6 mm. for the largest hard-stage male.

The largest soft-shelled male measured 4.2 mm., but Stauber reported one measuring 4.8 mm. With some reservation he referred males of this type to a second stage following the hard stage as he pointed out that they could also be abnormal crabs. This could be due to some sort of parasitism as reported for *P. pisum* by Mercier and Poisson (1929). When these authors found two soft-shelled males they naturally regarded them as abnormal because they differed from the (hard stage) males normally found, and finding that these crabs were infested by the parasitic isopod, *Pinnotherion vermiforme,* they concluded that here was the cause of it. Furthermore, since the two mentioned males as well as an infested, normal male were larger than the uninfested males in their material, they also concluded that the parasite causes an increase in the size of the host. Later, however, Atkins (1933) thoroughly studied the same parasite and found that none of the 8 soft-shelled males in her material were parasitized, but, since such males were scarce, Atkins still regards them as being abnormal. She points out that the parasitized males found by Mercier and Poisson were not larger than many normal (hard stage) crabs. In view of our findings, it is obvious to conclude that these soft-shelled males are normal pre-hard crabs, but, for certain reasons given in the discussion, the possibility that a hard-stage male now and then moults into a soft-shelled crab cannot be omitted. The maximum size of pre-hard males given in Table II may therefore be too high. It should be noted, however, that Stauber's finding of a larger mean size for his soft-shelled than for his hard-stage males is probably due to a sampling error. His material included only 13 of the former specimens, and they were collected over a long period of time.

A few atypical crabs occurred in our samples which combined features from pre-hard and hard stage morphology. Some also had all the characteristics of the pre-hard stages except that the carapace did not yield to the touch. It was brittle, however, and cracked at the slightest use of force. It is hoped to return to the significance of these "abnormalities" in a second paper.

Hard stage

This is the stage described by Stauber (1945) as the invasive stage (Stage I). Many of its characteristics have already been given in the section on the true invasive stage, and there is little to add to Stauber's excellent description.

One point is of particular interest, *viz.* the two cylindrical rods connecting the dorsal and ventral sides of the body. The diameter of these structures is the same as that of the spots on the sternum, while the dorsal spots, as noted by Stauber, usually are somewhat larger and more oval in shape. In proportion to the size of the crab the diameter of a single rod is equivalent to between ¼ and ⅕ of the width of the carapace. Thus the rods account for a considerable part of the endophragmal skeleton. The rods are firmly embedded in the sternum but disconnect rather easily from the carapace.

The hard stage differs markedly from the equivalent stage of *P. pisum,* specimens of which we have had the opportunity to examine. The latter have an arched carapace, possess no spots or rods, and are equipped with long, plumose swimming hairs on all walking legs. Also in contrast to *P. ostreum* the fourth and fifth pairs of pereiopods appear to be the main appendages used in swimming (Darbishire, 1900).

Female hard-stage crabs ranged in size from 1.3 to 2.7 mm., thus slightly extending the range of 1.4 to 2.4 mm. found by Stauber. However, two abnormal females were found which measured 4.1 and 4.6 mm. They had evidently been retarded in development for one reason or another since one had precocious gonadal development with abnormal, gnarled pleopods, and the other had hairy, biramous pleopods which normally do not occur before the crab has moulted into the third stage.

The smallest hard-stage male measured 1.5 mm. or the same minimum as found by Stauber. The upper size range, however, was considerably extended. The largest specimen found by Stauber measured 3.4 mm. while we found many exceeding that size, the largest measuring 4.1 mm. Furthermore, the Bivalve Laboratory possesses a male collected by Mr. Franklin Flower in Delaware Bay on December 18, 1952 which we found to measure 4.6 mm. The fact that Stauber found a soft-shelled male measuring 4.8 mm. seemingly indicates that even larger hard-stage males may occur. As discussed earlier, however, such large soft-shelled males may be abnormal.

It was found that crabs which had reached the hard stage in their first fall, that is, less than two months after invasion of the host, were somewhat smaller on the average than those which had over-wintered in a pre-hard stage and did not develop into the hard stage before growth and development had commenced again the following spring.

Post-hard stages

These are the soft-shelled female growth stages described by Stauber (1945) as the second, third, fourth, and fifth stages, the latter being the mature crab.

They have a thin, membranaceous and rounded carapace which yields to the touch. The slender pereiopods are subcylindrical and possess no swimming hairs. The four stages are primarily differentiated from one another on the basis of the stage of development of the pleopods and the proportional width of the abdomen (Table II).

Stauber's second stage is not clearly defined as his material included a number of pre-hard crabs. Thus he mentions (p. 282) a specimen measuring only 0.9 mm. which could not possibly have been a second stage crab. This mistake was due to the firmly established belief that the hard stage was the invasive stage. Stauber's figures of the second stage, however, agree well with the morphology of the second stage crabs reared by us from the hard stage in the laboratory.

Judging by our data it seems certain that the minimum size of the second stage cannot be less than 1.3 mm. The maximum size, as well as the size ranges of the following growth stages in our collections, agrees fairly well with the figures given by Stauber, except for the fifth stage. Here we found a size range of 4.4 to 15.1 mm. as compared to Stauber's figures of 6.0 to 14.9 mm. All size ranges are given in Table II.

As also pointed out by Stauber, morphological variations occur, a fact which now and then makes it difficult to place a given crab in a certain growth stage.

INVASION OF THE OYSTER AND SURVIVAL OF THE EARLY STAGES

As anticipated by Stauber (1945), invasion of the oyster in Delaware Bay takes place during late summer and early fall. In 1955 the first invasive stage crabs were noted on August 17th, but no oysters had been examined especially for the presence of *Pinnotheres* prior to that date. However, a careful check made through the spring and summer of 1956 again revealed no invasive stage crabs before the middle of August, *viz.* on August 16th. Nevertheless, scattered invasions no doubt occurred earlier as 1st stage zoeae were present in plankton samples on July 2nd,

TABLE II

Post-planktonic developmental cycle of Pinnotheres ostreum, based on the combined data of Stauber (1945) and the present authors

Stage of development	Range in carapace width in mm.	Most important external morphological characteristics	Biological factors
Invasive stage (First crab stage)	0.59–0.73	Flattened carapace and pereiopods. Posterior margins of pereiopods thickened, 3rd and 4th pairs have plumose swimming hairs. Two small, white spots on carapace and on sternum. Carapace hard around these spots.	Free-swimming until invasion of host. After invasion it is found in all parts of water-conducting system of the host.
Pre-hard stages	Male 0.75*–4.8 Female 0.75*–2.7*	Rounded carapace. Thin, flexible exoskeleton. Slender pereiopods. No swimming hairs. Large females practically indistinguishable from 2nd stage crabs.	Found in all parts of the water-conducting system of the host.
Hard stage (I stage of Stauber, 1945)	Male 1.4–4.6 Female 1.3–2.7	Carapace flattened and very hard. Flattened pereiopods with posterior margins thickened and with plumose swimming hairs on 3rd and 4th pair. Two large, white spots on carapace and on sternum. Males larger on the average than females.	Found free-swimming and in all parts of water-conducting system of the host. Copulatory stage. Males die in this stage.
Stage II	1.3*–3.1	Rounded carapace. Thin flexible exoskeleton. Slender pereiopods. No swimming hairs. Abdomen wholly contained in sternal grove. No hairs on pleopods.	Never free-swimming. Predominantly, possibly always, found only on the gills of the host.
Stage III	2.6–4.4	Edges of abdomen extend beyond depression in sternum. First two pairs of pleopods clearly segmented and supplied with a few hairs.	Only found on the gills of the host.
Stage IV	3.6–8.9	Relative width of abdomen larger than in preceding stage, just reaching coxae of pereiopods in most cases. Pleopods almost fully developed and well supplied with hairs.	As in 3rd stage.
Stage V (Mature female)	4.4–15.1	Abdominal edges covers coxae of pereiopods. Pleopods fully developed. The orange gonads may be seen through the thin carapace.	As in 3rd stage.

* Approximate measurements.

and the laboratory studies by Sandoz and Hopkins (1947), as well as our field data, show that only about 25 days or less are required from hatching to the development of the first crab stage, now known also to be the invasive stage. Invasions of oysters in Delaware Bay prior to the middle of August are, however, with little doubt on a very limited scale, at least in years with normal environmental conditions. This is also indicated by the fact that a distinct peak period of invasions occurs in early September. On August 22nd in 1956 only 3 crabs were found in 244 spat collected at Pierces Point, while 136 crabs were found in 199 spat collected on the same ground on September 23rd. Also, of 279 crabs collected there on September 17, 1955, 244 were still in the invasive stage, which indicates that a very recent mass invasion had taken place.

Since the peak period of oyster setting in Delaware Bay generally is in July, most spat will have grown sufficiently large to harbor one or more crabs by the peak of crab invasions.

It is not clear how late in the year invasions may occur as a few invasive stage crabs were found in oysters during all of the winter months. However, since growth and development stops about November 1st (Fig. 1), these crabs were probably late invaders retarded in their development by winter conditions. In 1956 a few ovigers[3] were collected as late as the middle of October. And in 1942, Stauber (1945) collected an ovigerous female as late as October 19th. The embryos were then almost ready to hatch and the first zoeae were liberated 4 days later. Whether the zoeae are able to carry through metamorphosis to the first crab stage that late in the year is perhaps doubtful. The bottom water temperature of Delaware Bay generally falls to about 15° C. by November 1st and to about 5° C. by December 1st, and as it appears that the young immature crabs do not grow and develop at temperatures below the first mentioned level (Fig. 1), the larvae probably do not either.

Surprisingly small spat may be invaded. Thus infested spat of less than 10 mm. in length were often found, and in one case a spat measuring only 4.2 mm. contained two crabs. Up to 7 invasive stage crabs were found in a single spat.

Stauber (1945) observed hard-stage crabs attached to the margin of oysters with their posterior ends towards the bill. This same orientation was also noted for the invasive stage in our laboratory experiments. As free-living crabs are also known to enter enclosures backwards, *Pinnotheres* probably enters its host with the posterior end first.

Once the crab has successfully invaded its host it may be found anywhere in the water conducting system of the oyster where it may stay while developing through to the hard stage, while later stages are found only on the gills. Next to these, the promyal and suprabranchial chambers are the areas usually inhabited by crabs of the early stages.

A preference to invade spat and, secondarily, yearlings rather than older oysters seems apparent from several types of observations. On August 23, 1956 only a single 1956 crab was found in 684 yearling oysters collected in the Cohansey River Cove while the few spat present were all infested, a couple of them with more than one crab. The only extensive comparative data, however, are from the Bay Shore Channel Bed where there was a heavy set of both oysters and crabs in 1956 as there had been in 1955. Two collections, each consisting of three different age groups of oysters were taken (Table III). One was taken on September 12th during the peak invasive period and the other was taken on October 9th. The oysters were all collected in the same dredge hauls, and nearly all the spat were taken directly from yearlings and older oysters.

---

[3] Oviger = ovigerous female. Term adopted from Ryan (1956).

As seen in Table III, only 21.5% of the older oysters were infested with 1956 crabs on September 12th, while 54.6% of the yearlings and no less than 76.7% of the spat were infested with crabs of that year class. On October 9th the differences were not so striking, a fact to which we return later.

A good number of the yearlings and older oysters were already infested with mature crabs when the 1956 set of crabs occurred (Table III, column 4). This could possibly have been one of the reasons for the preference indicated to invade spat, since the latter for obvious reasons were not already infested. Oysters with and without mature crabs were, however, invaded by 1956 crabs to about the same extent.

Possibly the preference to invade spat is more apparent than real. Failure of hard-stage crabs to invade older oysters was in some cases noted by Stauber (1945), indicating that the invasion is not always easily accomplished. Even if it is, the yearlings and especially the older, larger oysters may possibly still be able to cope with a good number of the tiny invasive stage crabs by enveloping them in mucus and pass them out by ciliary action and clamping of the valves. *Mytilus edulis* has been observed to expel inserted megalopa of *P. pinnotheres* by Atkins (1955).

TABLE III

Comparison of infestations with P. ostreum in three different age groups of oysters on the Bay Shore Channel Bed during and after the main invasive period

Date of collection	Age group of oyster	Number of oysters examined	Per cent infested with 1955 or older crabs	Per cent infested with 1956 crabs	Per cent of 1956 crabs in hard or post-hard stages	Per cent oyster with two or more 1956 crabs
Sept. 12th, 1956	Spat	167	0.0	76.6	3.1*	31.1
	Yearlings	339	33.6	54.6	47.3*	15.3
	Older oysters	186	50.0	21.5	90.0*	4.3
Oct. 9th, 1956	Spat	180	0.0	77.2	20.9	7.8
	Yearlings	289	34.4	72.7	73.9	27.3
	Older oysters	117	51.3	52.1	82.6	15.4

* An asterisk indicates that only hard stage crabs were found.

A comparison of the data in Table III, column 5, reveals that the incidence of oysters infested with 1956 crabs rose considerably for the yearlings and older oysters between the two sampling dates while it remained practically constant for the spat. In fact, the absolute number of crabs decreased in the latter group while it increased even more in the other two age groups than the data in column 5 indicate. This is seen in column 7, which shows that the incidence of multiple infestations with 1956 crabs decreased sharply in the spat from 31.1 to 7.8% but increased in the yearlings from 15.3 to 27.3%, and in the older oysters from 4.3 to 15.4%. Considering that the crabs apparently prefer to invade spat these data seem somewhat contradictory. An analysis of these and other data indicates, however, that the apparent paradox can be explained as being due to a higher mortality rate in crabs invading spat than in those invading yearlings and older oysters.

As stated above, only the absolute number of crabs found in spat decreased while the percentage of spat infested remained constant. In other words, the decrease in number of crabs between the two sampling dates was apparently either due to the death or the migration of crabs from spat containing more than one crab. Losses among "single" crabs possibly also occurred; if so, they were made up for by intermittent new invasions of crabs. The same trend that only one crab will survive in the same spat was also noted in 1955 at Pierces Point as well as on the Bay Shore Channel Bed. Only during the peak invasive period in September could spat with 3 and up to 7 crabs be found. A few weeks later only double infestations could be found, and the incidence of their occurrence was less than 10%. By the end of February, 1956 practically no spat contained more than one crab, and even a good number of "single" crabs apparently died during that month (Table I, Fig. 5). These mortalities came after a prolonged period of very low temperatures (Fig. 1), thus indicating that even "single" crabs are easily endangered by adverse environmental conditions.

The increase in number of yearlings and older oysters containing more than one 1956 crab between the two sampling dates (Table III) indicates in itself that the crabs survive better in these oysters than in spat. Further evidence of this is given in the same table (column 6). For both dates it is seen that a much lower percentage of the 1956 crabs had reached the hard and post-hard stages in spat than in the other oysters. This fact, together with the data given on crab-host size relationship in a later section, clearly shows that the crabs grow and develop considerably slower in spat than in larger oysters. In other words, the crabs thrive better in yearlings and older oysters, and it is therefore not surprising that they also survive better. The reasons for this will be discussed later. It is stressed, however, that a quicker rate of growth and development is probably in itself of prime importance for the survival of the crab during its first fall and winter since it is very likely that the earliest stages cannot withstand adverse conditions as well as later stages.

In summing up the data in Table III it may be concluded that intermittent invasions of crabs between the two dates, and a higher mortality rate of crabs invading spat than of those invading other age groups of oysters, constitute the main reasons why the yearlings and older oysters in direct contrast to the spat showed an increase in incidence of infestation on October 9th.

It was also considered whether the data in Table III could be at least partly explained by migrations of hard-stage crabs from spat to other oysters. However, since all the observed differences between the two sampling dates can be explained otherwise, while migrations could explain only little of it, the latter probably did not take place to any large extent. Furthermore, the available data indicate that the "loss" of crabs inhabiting spat occurred at a time when only few of these crabs had developed into the hard stage, *i.e.,* before these crabs were capable of migrating.

Growth and Development

Unless otherwise stated the following statements and discussion of results are based on studies of populations of *Pinnotheres* growing and developing in spat. This point should be kept in mind, because, with other factors being equal, growth and development would differ, depending on the size and age composition of the host population of oysters.

Growth curves, as represented by the mean sizes of all the crabs and their host oysters for each sampling date at Pierces Point and the Bay Shore Channel Bed, are plotted in Figure 1, together with a curve showing bottom temperatures at the latter station. The numbers of oysters and crabs on which the data plotted are based are listed in Table I. Both grounds were not sampled through the entire collecting period from September 17, 1955 to October 9, 1956, but there was a considerable period of overlapping between the beginning of sampling on the Bay Shore Channel Bed and the termination of sampling at Pierces Point.

On the first collecting date in September, 1955, 87.5% of the crabs found were still in the invasive stage, thus indicating that the spat had become infested very recently. On the other hand, since the incidence of infestation was higher than on any subsequent sampling date at Pierces Point it may also be concluded that the peak invasive period had terminated.

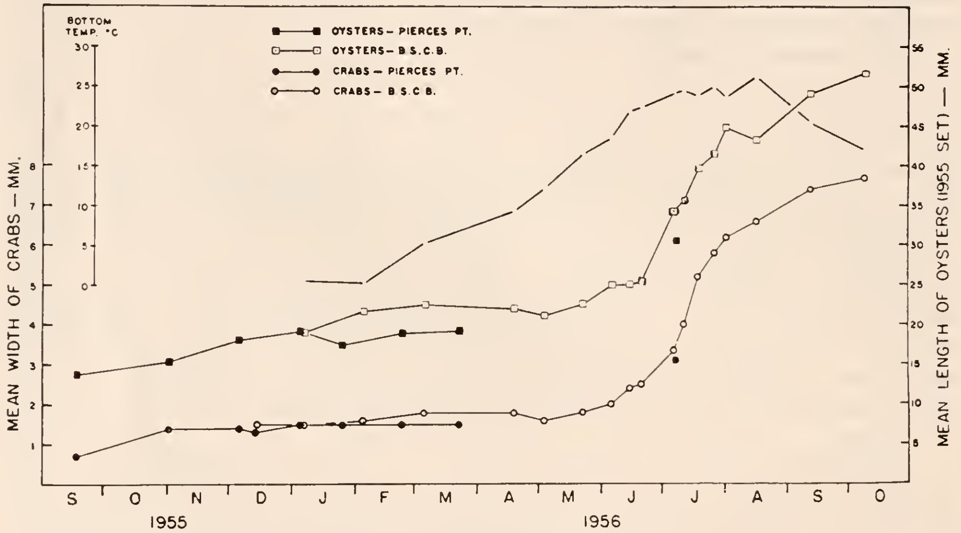


FIGURE 1. Growth of *Pinnotheres ostreum* (from the first crab stage to the mature stage) and their host oysters in Delaware Bay from September 17, 1955 to October 9, 1956. The data illustrated are based on the regular collections of oysters and crabs listed in Table I. Bottom temperatures for each sampling date on the Bay Shore Channel Bed are shown in upper curve.

Growth and development of the crabs took place at least until the beginning of November when bottom temperatures began to drop below 15° C., reaching the 5° C. level on about December 1st. During this initial growth period nearly all of the crabs developed into pre-hard stages. A few developed into the hard stage, and a few females even developed into the second and third stages. Throughout the winter months, however, there was a virtual cessation of growth and development. The large majority of the crabs overwintered in a pre-hard stage, the mean size of all crabs found in each winter collection staying below 1.8 mm. Renewed growth and development began about the middle of May when bottom temperatures again rose above 15° C. From about the middle of June the rate accelerated, the females going through the post-hard stages to the fully mature fifth stage with great rapidity. By July 18th all but one of the females collected were post-hard crabs, no less than 62.7% of them having already reached the final stage. By August 16th, 95.5% of the collected females were mature, more than half of them being ovigers. Thus, as suspected by Stauber (1945), *P. ostreum* reaches maturity in Delaware Bay within its first year.

The remarkable correlation between the growth curves of the crab and host populations in Figure 1 should be noted, although it is not so surprising considering that the crab is dependent on the food gathered by its host.

Males live only one year or less. One of the curves in Figure 5 shows the percentage of crabs in the hard stage, and another shows the sex ratio (as % females) for each collecting date on the Bay Shore Channel Bed. It will be seen that the majority of the crabs developed into the hard (copulatory) stage within a limited period of time around June 20th whereupon the males began to disappear from the oysters. Until and including that date, about 45% of the crabs were males but by July 26th, the percentage was only 6.7, and by September 12th no 1955 males could be found. It is shown in a later section that the males left their hosts in search of females in other oysters, and there is every indication that they did not commence a longer free-living life but died shortly after copulation with one or more females. All the above facts also constitute an important part of the evidence which shows that the males normally do not develop beyond the hard stage, *i.e.*, that no true second stage male exists.

The females continue to grow after they have hatched their first batch of eggs during the summer. The mean size of the 1955 crabs, all mature females, collected from yearlings on October 9, 1956 was 7.68 mm. with a size range of 5.2 to 10.0 mm. And this mean, as well as the maximum size, was the highest recorded for any sample taken from yearlings in 1956, no further samples having been taken after that date (Table I). Since we know that a positive crab-host size relationship exists, these figures do not necessarily apply to crabs invading oysters other than spat. However, the mean size of fourth stage crabs taken from older oysters was less than 6 mm., and we never found one as large as 8.9 mm. as Stauber (1945) did. This indicates that only few females will reach a size exceeding 10 mm. in their first year even in older oysters. And it seems almost certain that none will attain this size before growth is renewed after hatching of the eggs. How soon this growth commences is not known, but Freame (1943) observed an ovigerous *Pinnotheres* sp. moulting 10 days after hatching of the last eggs in the laboratory. Since very few one-year-old ovigers occur before the middle of July and since the egg-bearing period is 3 to 5 weeks, it is clear that all females collected in spring and early summer which had a size exceeding 10 mm. must have been crabs in their second (or third) year. The latter possibility is indicated by the fact that very large crabs my be found in older oysters just prior to, but before, growth begins in the spring. Berner (1952) also found that female *P. pisum* kept in aquaria became 3 years old.

Some indication of the rate of growth after the first year may be had from the data presented in Figure 2. It shows the size distribution of all ovigers found in two age groups of oysters, *viz.* yearlings (1955 spat) and older oysters, from collections made in August, 1956 on the Bay Shore Channel Bed. Judging by the difference in mean size of the two groups of crabs, an average increase in carapace width of about 3.5 mm. after the first year of growth is indicated. However, while the crabs collected from yearlings represented a true one-year age group, a number of the ovigers found in the older oysters were no doubt also only one year old, as judged by their relative small size and the fact that 16 immature crabs were also found in these oysters. This factor is no doubt the cause of some of the overlapping in size distribution of the two groups of crabs. The actual mean increase therefore probably amounts to at least 4.0 mm. Most of this growth probably takes place before the crab is two years old.

As stated earlier, a few of the crabs invading the 1955 spat developed as far as the third stage before growth was terminated by winter conditions. In the fall of 1956 this was the case to a much larger extent with 1956 crabs invading spat of that year class. Of 158 crabs collected from spat on the Bay Shore Channel Bed on October 9th, 26 had developed beyond the hard stage, one of them having reached the fourth stage. Forty-five of the crabs were too small to be sexed, but of the remaining, only 38 were males as opposed to 75 females. This indicates that the 26 post-hard crabs represented about 25% of all the females present. In contrast, only nine post-hard crabs were found among 205 crabs collected from spat on the same ground as late as December 14th in 1955. Indications are that the spat grew faster in the fall of 1956 than in the preceding year. This may well account for the observed differences, considering that the growth (and development) of a *Pinnotheres* population is correlated with the growth of the host population (Fig. 1). The latter fact will be demonstrated on an individual basis in the next section.

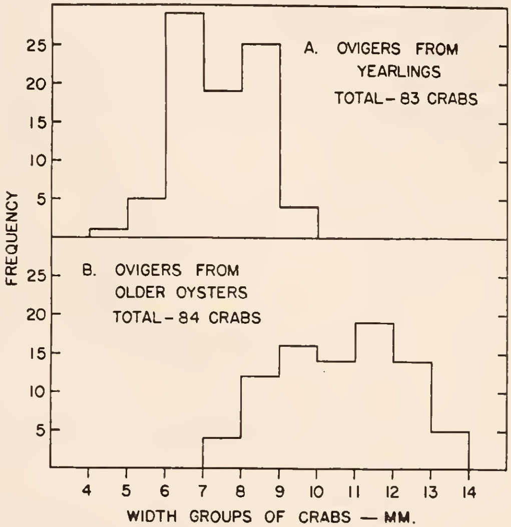


FIGURE 2. Size distribution of (A) ovigers collected from yearling oysters, and (B) ovigers collected from older oysters. Both groups of oysters were collected on August 1st and 16th in 1956 on the Bay Shore Channel Bed.

GROWTH AND DEVELOPMENT IN RELATION TO SIZE OF HOST

Atkins (1926) found a rough size relationship between 34 *P. pisum* and their host mussels in spite of the fact that the author was dealing with different age groups of both crabs and mussels. The latter was also true for the material used by Wells (1940) who, nevertheless, could demonstrate a clear size relationship between *Fabia subquadrata,* of the same sub-family as *Pinnotheres,* and its host *Modiolus modiolus.*

The following information is all based on data from collections of 1955 crabs from 1955 spat, which were taken in 1956 before the 1956 year class of crabs appeared, thus insuring that we were dealing with a known year class of both crabs and oysters.

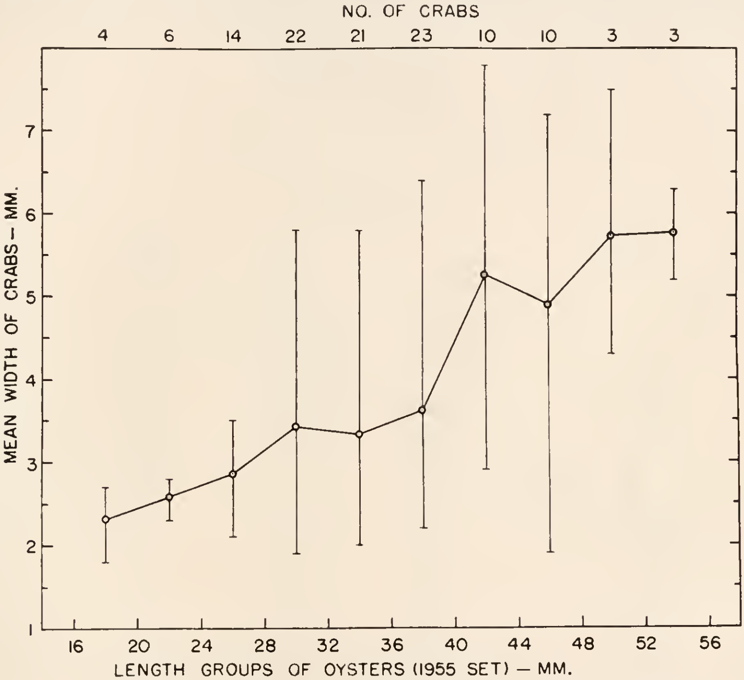


FIGURE 3. Crab-oyster size relationship. The mean width and size range is shown for all crabs, irrespective of stage, found in each 4-mm. length group of yearling oysters collected on the Bay Shore Channel Bed on July 6, 1956. All these crabs were for obvious reasons of the 1955 year-class.

In Figure 3, the mean sizes and size ranges of all crabs, irrespective of stage, found in each 4-mm. size group of oysters have been plotted for a collection made on the Bay Shore Channel Bed on July 6, 1956. A definite positive correlation between crab and oyster size is clearly present.

Plottings of the same kind, as well as statistical analysis, were undertaken for several other regular samples and they all show the same size relationship. It is not absolute, insofar as small crabs may well be found in large oysters, but the opposite is not the case. In other words, the factor or factors which limit the growth of the individual host also directly or indirectly limit the growth of the crab. Probably the most important factor is the amount of available food, which largely depends on the environmental conditions surrounding the individual host oyster.

While *growth* of the crab is retarded in slow-growing spat, there is evidence to show that *development* is not affected to a comparable extent. It may be safely assumed that having moulted into the fifth stage the females do not moult again before they have hatched their first batch of eggs. This means that any sample of mature females collected from yearlings before September will contain only an insignificant number of crabs which have grown since moulting into the fifth stage. In spite of being in the same stage of development, crabs from such samples vary, however, in size to an extraordinary degree. This, among other facts, is illustrated in Figure 4, which is based on the largest sample of young fifth stage crabs taken on a single date. The data presented reveal the same positive crab-host size (and growth) relationship as was demonstrated above on the basis of samples containing crabs of nearly all developmental stages. It appears, therefore, that the females simply develop into the fifth stage at a smaller size in smaller, slow-growing oysters than they do in larger, faster growing specimens. A further example of this is given in Figure 2. As discussed earlier, some of the smaller ovigers collected from the older oysters were no doubt only one year old. Yet none of them measured less than 7 mm. while the one-year-old ovigers taken from the yearlings comprised many specimens smaller than this. It should be stressed, however, that our data only point to the conclusion that the growth of the crab

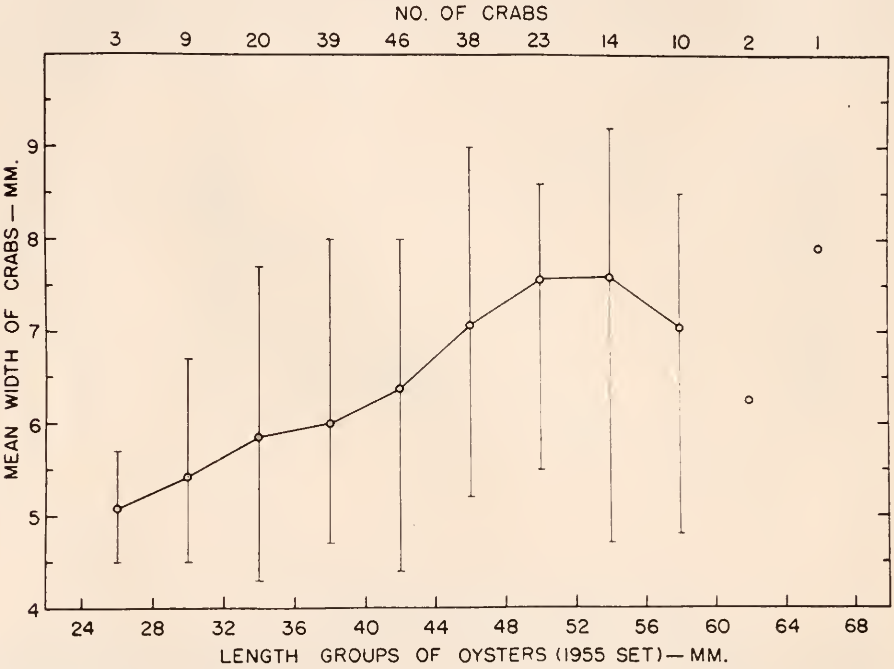


FIGURE 4. Size relationship between crabs in the same stage of development and their host oysters. The data, which are plotted as in Figure 3, are based on all fifth stage crabs collected from yearling oysters in the Cohansey River Cove on August 23, 1956. These oysters had been transplanted from the Bay Shore Channel Bed in early July.

is more affected by environmental conditions than is the development. Naturally the time element involved in the latter will also be affected under adverse conditions such as an insufficient food supply. A good example of this is given by the difference in rate of development of the young crabs in 1955 and 1956 which was mentioned in the preceding section.

GROWTH AND DEVELOPMENT IN RELATION TO MOULTING

Information on the size increase after moulting of individual crabs was obtained in two ways. In some cases the old exoskeleton was still present, together with the moulted crab, in oysters brought into the laboratory. In other cases, moulting occurred in the laboratory from crabs of known stage, sex and size placed in oysters by the method previously described.

TABLE IV

Moulting in Pinnotheres ostreum

Date of moulting	Sex of crab	Carapace width in mm. and stage of crab		Increase in carapace width in mm.	Increase in % of original carapace width
		Before moulting	After moulting		
17– 9–55	?*	0.66 I.S.	0.76 P-H.	0.10	15.0
22– 8–56	?	? P-H.	0.95 P-H.	—	—
10– 9–56	M.	2.90 P-H.	2.90 P-H.	0.00	0.0
20– 8–56	M.	4.10 P-H.	4.00 P-H.	−0.10	—
3– 9–56	M.**	3.30 P-H.	3.30 H.	0.00	0.0
8–10–56	F.	1.70 H.	1.95 II.	0.25	14.5
16– 7–56	F.**	1.80 H.	1.80 II.	0.00	0.0
4–10–56	F.	1.85 H.	2.15 II.	0.30	16.0
8–10–56	F.	1.90 H.	2.25 II.	0.35	18.5
6– 7–56	F.*	2.05 H.	2.40 II.	0.35	17.0
27– 7–56	F.*	5.40 IV.	6.10 IV.	0.70	13.0
30– 7–56	F.*	5.40 IV.	5.90 V.	0.50	9.0
?– 7–56	F.*	5.50 IV.	7.00 V.	1.50	27.5
9–10–56	F.*	9.90 V.	11.50 V.	1.60	16.0
9–10–56	F.*	11.90 V.	11.90 V.	0.00	0.0
6–10–56	F.	12.80 V.	13.30 V.	0.50	4.0

mAux

Symbols: I.S. = Invasive stage. P-H. = Pre-hard stage. H. = Hard stage. II., IV. & V. = 2nd, 4th & 5th post-hard stages.

Crabs marked with one asterisk moulted in nature.

Crabs marked with two asterisks had not hardened properly after moulting when the measurements were taken.

The laboratory experiments were performed primarily to secure: 1. Moulting of large soft-shelled males (into the hard stage) to test field information that these are pre-hard stage crabs. 2. Eventual moulting of hard stage males as a control for (1). 3. Moulting of hard stage females to secure known second stage crabs. As was expected, moultings of types (1) and (3) occurred while all hard-stage males died after some time.

All available data are given in Table IV, in which it may be noted that in some cases there was no apparent increase in carapace width after a moulting had occurred. This is probably due to the fact that some recently moulted crabs were preserved too soon after moulting and consequently the body had not had time to assume its normal shape. The poor food supply in the aquaria was no doubt also partially responsible for no size increase. If these moultings are disregarded, the data indicate a mean increase in carapace width of about 15% after each moult, regardless of the previous size and stage of the crab. If this be true, it would take about five moults for the invasive stage crab to develop through the pre-hard stages into the hard stage. Preliminary morphometric studies of pleopod length and development in relation to body size of our collection of pre-hard stage males seem to verify this. Supporting evidence is the fact that a pre-hard stage crab measured only 0.95 mm. after it had moulted from another pre-hard stage which was no doubt the stage immediately following the invasive stage. Since pre-hard and hard-stage males are larger on the average than females of the equivalent stages, differences may exist in the average number of moults and size increase in the two sexes. More detailed studies are necessary to check on these points.

Only one of the nine fairly large pre-hard males used in our experiments moulted into the hard stage, while two others moulted without a change of stage. Although the former was only slightly larger than the mean size of Stauber's 13 "2nd stage" males, the moulting still serves as a support of other evidence previously referred to, that no true second stage exists in the male. More important is perhaps the fact that all hard stage males brought into the laboratory died within a few weeks while hard and post-hard stage females could be kept alive for many weeks, even in the less favorable environment of the Petri dishes, and with a very poor food supply. Moulting experiments were done with seven hard stage males, none of which moulted, nor did other hard stage males in Petri dishes. In contrast to this, four out of six hard stage females inserted into oysters moulted into the second stage (Table IV), indicating that there was no reason to suspect that hard-stage crabs for one reason or another were unable to moult under laboratory conditions. Nor is further development of the hard stage dependent on copulation as suggested by Orton (1921) and Atkins (1926) for *Pinnotheres pisum* (see next section).

Moultings of fifth stage crabs were noted several times by Stauber (1945) as well as by the present authors. In view of the mean increase in carapace width of about 4 mm. or more which takes place after the crab has first developed into the fifth stage it seems likely that at least three moults occur in this stage before the maximum size is reached. Since the crab may develop into the fifth stage at a size anywhere from 4.4 to 8.9 mm. (Table II), depending on environmental conditions, the number of these moults must, however, vary to some extent.

It seems probable that growth moultings with little or no morphological changes may occur in all stages with the exception of the invasive stage and probably also the hard stage. Such moultings probably explain why a few crabs, being intermediate in character, could not be placed with certainty in one or another of the otherwise well defined growth stages.

Since *Pinnotheres* copulates precociously it differs from most brachyurans in moulting between copulation and egg-deposition. As the female usually receives sperm while in the hard stage at least four such intermediate moultings may occur.

Copulation, Egg-Deposition, Hatching, and Larval Life

Thompson (1835) assumed that the male of *Pinnotheres pisum* goes from host to host in search of females during the copulatory period. This was verified by Orton (1921) who found males trapped between the valves of the host, *Mytilus edulis*. He also found a hard stage female with its spermathecae filled with mature sperm, thus showing that this crab copulates at an extremely early age. Sperm was also found in the spermathecae of later stages, including the fifth stage. Atkins (1926) corroborated Orton's findings, but she also found a few fifth stages without sperm, and this led her to state that a second copulation possibly took place. Stauber (1945) doubted that this could be the case in *P. ostreum* because it would involve copulation between crabs which in his opinion differ too much in size. It may here be mentioned that the male of *P. pisum* is considerably larger on the average in relation to the size of the female than is the male of *P. ostreum*. Berner's (1952) paper, which contains many statements on the biology of *P. pisum*, unfortunately lacks vital information on materials and methods, and the author has taken no notice of later papers on the subject than that of Orton (1921). He states that the free-swimming male seeks mussels containing females which are about one year old, and that after copulation, the male again leaves the host as the ovigers are always found alone. The author refers to Orton's finding of precocious copulation, but also states that copulations involving mature females took place in his laboratory tanks. It appears, however, that this statement is based solely on the author having seen males close to females, and the occurrence of egg-deposition by females long after they had been collected. A number of other papers contain valuable contributions on the reproductive biology of pinnotherid crabs, but they are best dealt with in connection with our own observations.

In the present studies an attempt was made to gain information on the reproductive activities of a known year-class of crabs from a single locality, thereby eliminating as many variable factors as possible. Delaware Bay proved to be particularly well suited for this purpose since the extreme variations in water temperature between the seasons of the year give rise to distinct, short peak periods of the different phases involved.

Most of the information gained is illustrated in Figure 5. The data plotted are based on the material from regular samples taken on the Bay Shore Channel Bed, consisting of 1955 crabs from 1955 spat (Table I), and an extra sample (see figure legend).

One of the curves (Hard crabs) shows that the percentage of hard-stage crabs remained at a consistently low level throughout the winter and spring, *viz.* during the period in which it was shown earlier (Fig. 1) that no growth took place. In June, however, the large majority of the crabs developed into the hard stage almost simultaneously. A distinct maximum occurred around June 20th, when about 66% of all the crabs collected were hard-stage crabs. Many of them had a soft carapace, indicating a very recent moulting into the hard stage.

Another curve (Female crabs) shows that on the same date there were still as many males present as in all earlier samples, *viz.* about 45% of all crabs collected. After June 20th, however, they began to disappear rapidly, as evidenced by the rising percentage of females in each subsequent sample. Towards the end of July, less than 5% of the collected crabs were males, and by early September not a single 1955 male could be found.

Also beginning on about June 20th, the incidence of infestation (Oysters infested), which had remained at a constant level, around 58%, since February, began to fall quickly. It continued to do so until the number of males occurring in the samples became insignificant. Then the incidence again became constant, but now at the lower level of about 35%, or just about the level where it would have been on June 20th if only the females had been considered.

A fourth curve (Double infestations) in Figure 5 shows that within the same period of time, beginning towards the end of June, a notable number of double infestations began to occur. Those shown for the winter months were due to original multi-infestations by invasive stage crabs, but those which occurred during the summer could only be due to new invasions by hard stage crabs. An examination of this summer material showed that each double infestation consisted of one crab of each sex. Such pairs were also found in collections made on other grounds during this period of the year. In a few cases two or three males had invaded the same oyster containing a female. It was significant that the males and females making up these pairs nearly always were found close together on the gills of the host while the crabs found together in the same host during the fall and winter nearly always were found widely apart from one another.

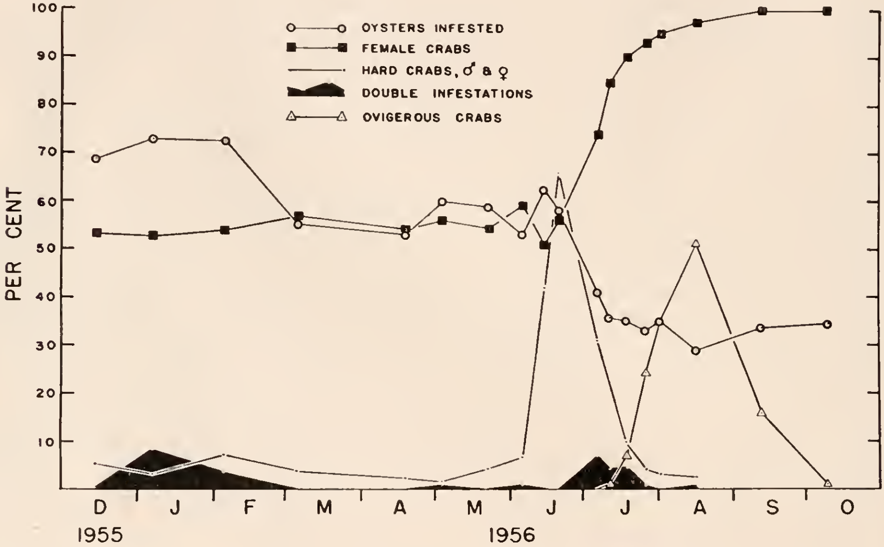


FIGURE 5. The curves show: 1. Incidence of infestation and double infestations of the 1955 year-class of oysters on the Bay Shore Channel Bed, and 2. Sex ratio (as per cent females) and development (in part) of the 1955 year-class of *P. ostreum* inhabiting these oysters, in the period from December 14, 1955 to October 9, 1956. Data on double infestations include results obtained from extra sample of 334 infested spat collected on July 11th, 16 of which were double infested.

All these facts, illustrated in Figure 5, can be interpreted only in one way, *viz.* that having developed into the hard stage, the males left their host to search for females in other oysters. Having copulated with one or more females they probably died within a few weeks or less. The latter statement is based also on the fact that a few of the males found together with females were already dead.

Even before the males began to disappear we generally found somewhat fewer males than females. For this reason, and because a number of males may be devoured by predators during their temporary free-swimming existence, it seems reasonable to deduct that a male will copulate with more than one female. At least there is every indication that all females become ovigerous in their first summer and this could hardly be accounted for otherwise.

Of 33 pairs found in yearlings from the Bay Shore Channel Bed in July, 1956, 32 of the males were in the hard stage, while 21 of the females were either in the hard or the second stage. The remaining females were all in later stages of development, a few having reached the fifth stage. This does not necessarily mean, however, that males can or do copulate with post-hard females. Possibly they may live up to a few weeks after copulation, staying in the last oyster they visit, while the female continues development into later stages. The great rapidity of growth and development of the females in late June and in July (Fig. 1), and the fact mentioned earlier that some of the males found together with females were dead when collected, would support such a conclusion. The few of the hard and second stage females from double infestations which we examined had sperm in their spermathecae, a fact which also serves as circumstantial evidence leading to the conclusion that the females were probably all fertilized while in the hard stage.

Nevertheless, the above evidence is not conclusive, especially since other observations indicate that males may copulate with later stages. Of six known second stage crabs of the 1956 year-class examined in the fall of that year, only one contained sperm. Theoretically such females could of course remain unfertilized or become capable of producing infertile eggs only, but this does not seem a likely explanation. In the laboratory we also once observed a hard stage male, with its pleopods extended, enclosed under the abdomen of a fifth stage female. However, whether an actual copulation took place or not, we do not know. Berner's (1952) statement that female *P. pisum* may be fertilized in the fifth stage is not based on conclusive facts, which does not mean that it could not be true. The author did not observe an actual copulation, and the crabs which became ovigerous in the laboratory tanks may well have had sperm in their spermathecae when they were collected (see below). More information is certainly needed to settle the question.

The observations referred to above enlighten, however, our understanding of another point of interest. Orton (1921), and also Atkins (1926) suggested that further development of the hard stage female of *P. pisum* depended on copulation. Since known second stage crabs containing no sperm were found, this cannot be true for *P. ostreum,* and there is little reason to suspect that different species of the same genus should differ in this respect.

Atkins (1926) stated that it was extremely probable that the first implantation of sperm was sufficient to fertilize several batches of eggs in *P. pisum*. That it is sufficient for at least the first two batches was actually shown by the same author in a later paper (Atkins, 1955). She kept several female *P. pisum* isolated in the laboratory, and one of these deposited eggs on September 27, 1952 and again on May 26, 1953. Our data point to the same conclusion for *P. ostreum*. Twenty-one mature females were examined during the fall of 1956. Of these all but one had sperm in their spermathecae, although all of them probably had been ovigerous during the preceding summer. Theoretically they could have received sperm from 1956 males, but since only one of the 1956 second stage females examined during the same period contained sperm, this seems extremely unlikely.

The fact that a fertilized 1956 female was found in the fall of that year reveals that copulation is not restricted to the early summer as the data in Figure 5 so strongly indicate.

This poses the question whether males which copulate in the fall may live through the following winter and perhaps copulate again the following early summer. In this respect it may be significant that there were fewer males than females in our regular samples from the Bay Shore Channel Bed even before the males began to disappear entirely. Sampling on that ground did not begin before December, that is, after a probable initial copulatory period was terminated by winter conditions. The "deficiency" of males may therefore have been due to a natural death of males after copulation in the fall. Some of them may also have fallen prey to predators while moving from one oyster to another in search of females. The case mentioned earlier where only 38 out of 113 sexable crabs, or 33.6%, were males points to the same conclusion. This collection was made from spat on October 9, 1956, and it will be recalled that a considerably higher percentage of crabs invading spat reached the hard stage before the onset of winter in 1956 than in 1955. There is therefore also little doubt that a higher percentage of males became sexually active in the fall of 1956 than in the preceding year. Hence, if the above hypothesis on the "missing" males is correct, one would also expect that a higher percentage of males died in the fall of 1956 than in the fall of the preceding year, and this is exactly what our data indicate. While we have good data on the 1955 year-class, this is not, however, the case for the 1956 year-class. The sex ratio of the latter group, 33.6% males, is based on the single collection from October 9th, a date which furthermore falls so early in the fall growing season, that changes may well have occurred before winter conditions stopped growth and development as well as sexual activities. Nevertheless, there can be little doubt that early summer is by far the main copulatory period for *P. ostreum* in Delaware Bay under normal environmental conditions.

While copulation may take place either in the fall or the following early summer, egg-deposition of crabs in their first year does not occur before early July. Thus, in 1956 the first oviger from a yearling (1955 spat) was taken on July 11th, and no oviger less than 10 mm. in size occurred in older oysters before July 6th. The last one-year-old ovigers were found on October 9th. The curve (Ovigerous crabs) in Figure 5 shows the percentage of 1955 crabs being ovigerous on all collecting dates on the Bay Shore Channel Bed. The highest incidence occurred in middle August when more than 50% of the collected crabs were egg-bearing females. This was also the case elsewhere in the Bay, and the following case may be mentioned. In early July of 1956 a large number of yearlings were transplanted from the Bay Shore Channel area to the Cohansey River Cove about 24 nautical miles farther up the Bay. Of 205 females collected from these oysters on August 23rd, nearly 60% were ovigers. Of these, 26% had dark brown eggs or had already begun to hatch zoeae which could be found in large numbers in the mantle cavity of oysters containing such crabs. Judging by the data illustrated in Figure 5 as well as other information, a female may copulate in the hard stage and develop into a fifth stage oviger within a period of four to six weeks. The smallest oviger found in our collections measured only 4.5 mm.

In their second (and third) year the females may become ovigerous somewhat earlier. This is probably because they do not have to utilize a great deal of food for rapid growth and development prior to the deposition of the eggs as is the case for the one-year-old crabs. Nor do they have to await the visit of hard stage males, which do not become abundant before sometime in June, as they already have sperm in their spermathecae. In 1956 several ovigers exceeding 10 mm. in size were collected from older oysters on June 14th, or about three weeks before one-year-old ovigers were found. Judging from our data derived from a material of older crabs collected at intervals from May 22nd to October 9th in 1956, the peak period of egg-deposition also occurs two to four weeks earlier than for one-year-old crabs. From a comparison of the size distribution within these samples it also seems that, although some crabs no doubt become three years old, many of them probably die after they have hatched their eggs the second summer.

The newly deposited egg mass is orange in color. It changes gradually from a deep orange to a light brown and finally to a dark brown color. The eggs measure about 300 microns in the hatching stage (Stauber, unpublished) or about the same as in *P. pisum* (Lebour, 1928). Ovigers measuring 9.4 and 10.8 mm. in width carried 7957 and 9456 eggs, respectively. An oviger of *P. pisum* in the Copenhagen University Museum, measuring 10.4 mm., carried more than 5800 eggs. Berner's statement that this species deposits about 100 eggs is therefore erroneous.

It is not known for how long the female carries its eggs, either in Delaware Bay or elsewhere. An ovigerous *Pinnotheres taylori* brought into the laboratory on March 16th (1933) did not hatch before the first week of May (Hart, 1935). The egg-deposition and hatching of six *P. pisum* was observed in the laboratory by Atkins (1955). She found that the egg-bearing period varied between 35 and 59 days, stating that temperature differences probably constituted the main reason for the notable time difference, and that the period would no doubt be shorter in nature. Atkins also brought a *P. pinnotheres* with eggs in the early stages into the laboratory, and here hatching occurred after 40 days. Our field data from Delaware Bay, partly illustrated in Figure 5, indicate a somewhat shorter egg-bearing period for *P. ostreum*. As noted earlier, the first one-year-old oviger did not occur before July 11th, or possibly July 6th, but the peak occurrence came already in middle August. The fact that Sandoz and Hopkins obtained zoeae from a *P. ostreum* carrying a bright orange egg mass after only 12 days in the laboratory points to the same conclusion. We believe the egg-bearing period in nature to be three to five weeks. While it is almost certain that only one batch of eggs is produced in the first spawning season, the possibility that some crabs may spawn twice in the second (and third) year cannot be omitted.

As part of the oyster research program in Delaware Bay in 1956, approximately one thousand plankton samples of a hundred liters each were collected from June 14th to September 12th. This gave us an opportunity to gain some knowledge on the occurrence of *Pinnotheres* larvae. All samples from the Bay Shore Channel Bed and within a radius of about 5 nautical miles were therefore checked for the presence of such larvae.

The first zoeae were found on July 2nd and the last on August 20th, but, while samples were collected almost daily up to the latter date, rather few samples were taken thereafter. However, four samples of a thousand liters each instead of the normal volume were taken on September 12th. In Chesapeake Bay, Sandoz and Hopkins (1947) found oyster crab larvae in the plankton from June through August, but they also took few samples outside this period. In our own samples, 137 first stage and one second stage zoeae were collected while none of the later stages, nor the megalopa, were encountered. Two invasive stage crabs were taken, however, one on August 31st and another on September 9th.

Fifty-four of the 137 first stage zoeae were found in a single bottom sample from the Maurice River Cove on July 11th. Since numerous samples contained no larvae, and since the remaining 83 specimens were collected in 35 different samples, the occurrence of so many zoeae in a single sample seems strange. It is therefore highly possible that the aperture of the sampling hose happened to pass close to an oyster containing a crab which was in the process of liberating zoeae.

Seventy of the 83 larvae mentioned above were caught between July 20th and August 20th. Although few samples were taken after the latter date, this still indicates a peak occurrence of first stage zoeae within the above period since the data correspond well with the finding of a peak occurrence of older ovigers in the latter half of July and of one-year-old ovigers in middle August, as well as with the known peak invasive period that falls in early and middle September.

Hart (1935) found that the first crab stage of *Pinnotheres taylori* emerged four weeks after hatching in the laboratory. Also in the laboratory, Sandoz and Hopkins (1947) observed the first crab stage of *P. ostreum* emerging about 25 days after hatching. No other species of pinnotherid crabs have, as far as we know, been reared to the first crab stage. Since data from the same ground (Bay Shore Channel Bed) and from the same year (1956) show (1) a distinct peak in number of ovigers in middle August, and (2) a distinct peak period of invasions in early and middle September, there is every reason to believe that the average length of larval life of the oyster crab in Delaware Bay does not exceed 25 days as found by Sandoz and Hopkins under laboratory conditions. It is probable that it is even shorter as judged from the data above and the fact that the larval development may be slowed down under laboratory conditions as pointed out by Atkins (1955). The above data and conclusions are admittedly based on one-year-old ovigers only, as older crabs deposit eggs somewhat earlier in the season. However, the former year-class constituted the large majority of the adult population in 1956, indicating that the peak invasive period was determined by invasive stage crabs deriving from the yearling crabs. Our data also indicate that the crabs invade a host as soon as they have developed into the invasive stage.

Although the first zoeal stage exhibits a distinct positive phototropism in the laboratory, only 18 of the collected specimens were taken in surface samples. This is in good accord with laboratory observations on other species of this genus. Lebour (1928) states that the newly hatched larvae of *P. pisum* at first rise to the surface but soon go to the bottom where they feed. The zoeae of *P. maculatus* seek the bottom after three to five days (Welsh, 1932), and those of *P. latissimus* do it after only one or two days (Miyake, 1935).

THE CRAB-OYSTER ASSOCIATION

Coupin (1894) discovered that *P. pisum* feeds on food filtered from the water by its host. This has been confirmed by later authors including Orton (1921) who, through "windows" in the host's valves, observed how the pea crab picks food strings from the margins of the gills with its chelipeds. MacGinitie and MacGinitie (1949) used the same method in observing the feeding activities of *Fabia subquadrata* (referred to as *Pinnotheres concharum*). Stauber (1945) found that *P. ostreum* feeds in the same manner but that it will also catch newly formed mucus-food masses with its walking legs, then reach beneath its abdomen with its chelipeds, comb the legs, and pass the food on to the mouth.

How young crabs feed when inhabiting parts of the oyster other than the gills is not known. MacGinitie and MacGinitie (1949) observed that the pea crabs *Scleroplax* and *Pinnixa* are able to filter food from the water by their feathery mouth parts. These crabs belong to another sub-family than *Pinnotheres* and may therefore differ from the latter genus in this respect. However, several species of *Pinnotheres,* such as *P. pugettensis, P. taylori,* and *P. pinnotheres,* live in the excurrent region of the atrial cavity of tunicates. And they must, as pointed out by Wells (1940), take their food from the water brought in by the host to serve as a source of food for the tunicate itself. The crabs can hardly do this in any other way than by filtering the water with their mouth parts. It is therefore very likely that immature *P. ostreum* may also feed by the filtering method. Such a feeding method could explain why a large number of immature crabs in a single oyster does not seem to affect the tissues of the host more than one crab as observed by Stauber (1945). However, this manner of feeding is probably not effective enough for the older stages. Post-hard stage crabs are found only on the gills, indicating that only the feeding on the food-laden mucus strings can secure the crab enough food for the rapid growth and development and the production of eggs which take place in late spring and summer. In any case, whether the young crabs feed by one or the other method or both, it is dependent on the amount of food particles brought into the oyster per time unit. It is therefore highly possible that the difference in survival of oyster crabs invading spat and older oysters may be due to the difference in amount of water pumped by the host animals.

The ordinary feeding activities of *P. ostreum* were found by Stauber (1945) to be harmful to the host, particularly in causing gill erosions. He described two types of gill damage, *viz.* the small-crab type with a local, sharply delimited erosion of one or more demibranchs, and the large-crab type where an extensive shortening of one or more demibranchs may be seen reaching from the anterior end of the gills to a point usually ventral of the adductor muscle. It is our impression that the gill damage gradually develops from the first type to the other. Nearly all infested oysters show some gill damage. Examination of 1502 oysters, all of the 1955 year class, collected from January 6th to August 1st in 1956 revealed that about 50% had light gill damage, about 40% had moderate gill damage, about 9% had heavy gill damage, and only about 1% had no discernible gill damage. Among older oysters we found a few extreme cases of heavy gill damage where there was hardly anything left of the gills, and such oysters were usually also very poor in condition.

Since the gill damage, as shown by Stauber, interferes with the feeding mechanism of the oyster, and since the crab feeds on food strained from the water by its host, the presence of a crab might be expected to interfere with the growth and reproduction of the host. Overcash (1946) studied quantitatively the condition of Virginia oysters. Employing an index based on the dry weight of the meat in relation to the volume of the shell cavity, she found that infested oysters were definitely poorer in condition, on the average, than oysters without *Pinnotheres.* Experimentally it has been shown by Egami (1953) that removal of gill tissue in the Japanese oyster, *Crassostrea gigas* causes a decrease in growth rate.

During the present studies the lengths of all infested as well as uninfested 1955 oysters collected on certain sampling dates in 1956 were measured to check for any possible differences in growth rate. The results are presented in Table V, and show no indication of any differences. However, the data pertain only to the possible effect on the oysters' first year of growth, a period in which it could only have harbored a mature crab for about 10 weeks or less. Furthermore, the data do not give any information on possible differences in increase of shell thickness or weight of the living tissues, a fact which should be considered since the correlation between shell and tissue growth is still obscure (Korringa, 1952). Very possibly only the presence of a mature crab over a longer period of time will interfere noticeably with the growth of the host under normal environmental circumstances.

Awati and Rai (1931) presented some very interesting data on the effect caused by *Pinnotheres* sp. on the sex ratio in populations of *Ostrea cucullata* in Indian waters. Among 794 uninfested oysters there were 41.7% males, 56.4% females, and 2.9% hermaphrodites, while in 86 infested oysters there were 82.6% males, only 10.4% females, and 7.0% hermaphrodites. Since females could be induced to change sex in the laboratory by simple starvation, the authors concluded that the pea crab probably interferes with the food intake of the oyster enough to induce it to produce sperm instead of the more "expensive" eggs.

TABLE V

Comparison of mean shell length of infested and non-infested 1955 oysters on the Bay Shore Channel Bed

Date of collection	Number of infested oysters	Number of non-infested oysters	Mean length in mm. of infested oysters	Mean length in mm. of non-infested oysters
6–1–56	120	48	19.1	19.1
5–6–56	103	91	24.3	26.0
6–7–56	116	166	34.3	34.7
26–7–56	118	239	41.6	41.6
12–9–56	54*	125*	49.2	49.7

* Infestations of these oysters with the 1956 year-class of crabs were not considered.

It would be of interest to know whether the sex ratio is also affected in the American oyster. The genus *Crassostrea* does not exhibit frequent, normal sex changes as does the genus *Ostrea,* but, nevertheless, it has a strong tendency towards protandric hermaphroditism, and the sex ratio is definitely influenced by environmental conditions (Amemiya, 1929; Coe, 1934). Further, Amemiya (1935), and also Egami (1953) have shown experimentally that removal of part of the gill tissue in groups of *Crassostrea gigas* causes the proportions of males to females to rise during the breeding season, *if* the operations are performed no later than the previous October. There is therefore reason to believe that the reproductive system of *Crassostrea virginica* may be affected by the oyster crab, at least in the second spawning season in which it harbors the same crab, *i.e.,* yearling oysters are probably never affected. This conclusion is also supported by the findings of Berner (1952) who examined the gonadal condition of more than 300 *Mytilus edulis* infested with *P. pisum*. He found that a partial or even a complete cessation in the production of sexual products often occurred in mussels containing a crab measuring 10 mm. or more in carapace width, but that mussels containing a smaller crab very seldom seemed to be affected. And according to the same author there can be no doubt that the larger crabs were all in their second or third year.

Discussion

Of primary interest in this study of *P. ostreum* was the revelation that both sexes invade their host in the first crab stage, which has become morphologically adapted for its dual role of swimming about in search of a host and of entering it. Further, that about four more, hitherto undescribed, growth stages follow this stage before the crab moults into what was until now thought to be the invasive stage, *viz.* the hard stage.

Since the crab is found in an animal that has been the subject of more research than most marine invertebrates, it may well be wondered why the presence of these early stages has so far been overlooked. There are, however, many reasons for this. They are not found throughout the year. In fact, in fast growing spat and in yearlings and older oysters they may develop into the hard stage within a few weeks. Under all circumstances many of them will soon reach a pre-hard stage in which, if at all possible, it takes more than a casual observation to distinguish them from second stage crabs. The firmly established belief that the hard stage was the invasive stage is in itself another explaining factor. Moreover, the small size and often concealed position of the early stages in all parts of the water conducting system of the host makes them easy to overlook. A careful microscopical examination of the oyster tissues is indeed necessary to find all crabs present.

It is well known that other species of *Pinnotheres* are found in a large number of common intertidal or littoral host animals all over the world, except in arctic and antarctic waters. In spite of this, no developmental stages, equivalent to the invasive or the pre-hard stages of *P. ostreum,* have been taken and recognized as such in nature. It is therefore of considerable interest to discuss the general conclusions concerning the life-history and biology of species of *Pinnotheres* that may or may not be drawn from a study of the literature and the results of the present work. In addition, some information from recent studies on *P. pisum* in Scandinavian waters will also be utilized although the materials and methods as well as the detailed results are not published yet.

More than one hundred species of *Pinnotheres* have been described (Rathbun, 1918; Tesch, 1918; and others), and they are doubtless all either commensals or parasites. This is well established for the large majority of the species, but in some cases no reference is made to a host for a particular species. In most of these cases, however, it can be ascertained that the author's material consisted of hard stage crabs, usually only males, and the collection of this stage free in the water obviously does not mean that the species concerned is free-living. In other cases the original collector has not bothered to note from which host the crab or crabs were taken.

Most of the species inhabit bivalves while a few are found in polychaete tubes, gastropods, holothurians, and tunicates. The majority of them are also known to occur in more than one host. Many have been taken from half a dozen different bivalves or more (Sakai, 1939; and others), and a few are even known to occur in widely different types of hosts. For instance, *P. maculatus* has been collected both from bivalves and *Chaetopterus* tubes, and *P. pinnotheres* occurs in bivalves as well as tunicates. One of the few species that may be host specific is *P. placunae* described by Hornell and Southwell (1909) which seems to be morphologically adapted to inhabit *Placuna placenta,* a bivalve with closely approximated valves. *P. ostreum* also occurs in other bivalves than *C. virginica* as it has been found in *Pecten* sp. by Ortmann (Gerstaecker and Ortmann, 1901), and on one occasion in *Anomia simplex* by the present authors.

Once the female oyster crab has developed beyond the hard stage it never leaves the host. During the many years of biological work on the Delaware Bay oyster beds, no mature or other post-hard crabs have ever been collected outside a host. We have found dead and dying oysters (gapers) containing post-hard, usually fifth stage crabs, some of which were also dead. It may be that neither the live nor the dying oyster open enough to enable the crab to crawl out. We are, however, convinced that a post-hard crab would not leave a live oyster even if it had an opportunity to do so, and this probably applies to all *Pinnotheres*-host associations. It is certain that adult females of species living in worm tubes, holothurians, and tunicates are unable to leave their host as is evident from the papers by Semper (1881), Enders (1905), Wells (1928, Fig. 76), Tu (1938) and others. The same must be true for those species which live in burrowing and boring bivalves. They can only be entered or left through the siphons which are not wide enough to admit the passage of a mature female. Wells (1940) has some data on this problem for other genera of pea crabs, but his results seem somewhat contradictory to one another. On the other hand, Tu (1938) writes that he has often seen *P. affinis* leave and re-enter the scallop, *Pecten hastatus?,* and Berner (1952) states that all stages except the larger ovigers of *P. pisum* freely change from one host to another. It is not clear, however, whether Tu's observations apply to the post-hard stages, and Berner's statement seems to be based more on opinions than on actual observations. In contrast to Berner, Thompson (1835) emphasizes that although he made extensive dredgings with fine nets, and at all seasons, on grounds with infested mussels he never found a free-living female *P. pisum.*

The species may of course vary in this respect, and it is obvious that some species of host animals would be easier to leave and enter than others. However, since the mature female with its soft-shelled carapace and feeble walking legs seems entirely unadapted for even a temporary stay outside a host, more convincing evidence seems needed to accept Tu's and Berner's statements. These crabs are only able to move slowly and they would be easy prey for any predators. They often lie on their backs on the aquaria floors as well as inside their host, and this is obviously a dangerous habit unless the crab is in a protected position. It may also be asked why two different stages, both specialized for swimming and entering the host, should have developed if all stages could freely move from host to host. In this connection we cannot accept Berner's view that the softness of the carapace facilitates the invasion of the host since even the smaller, specialized hard-stage crabs may be trapped and severely damaged between the valves (Orton, 1921). It is very possible, however, that some or all species inhabiting bivalves that gape upon death may leave their host under such circumstances. This was observed by Wells

(1940) for some other genera of pea crabs, but in view of the above we find it doubtful whether a post-hard *Pinnotheres* would succeed in finding and entering another host.

There can hardly be any doubt that all species of *Pinnotheres* invade their host in the first crab stage. Pre-hard stages must exist in all of them as evidenced by the large size of the hard-stage crab in the many species in which this stage is known, *viz.* all the species of which the male has been described. It is also reasonable to assume that they are soft-shelled and without swimming hairs as we have found for both *P. ostreum* and *P. pisum,* and that they are therefore not adapted either for a free-living existence or for invading the host. Hence the crab must invade its host either as a megalopa or in the first crab stage as we can safely disregard Semper's (1881) suggestion of the invasion taking place in the zoeal stage. Since it is now known that the two species mentioned above invade their host in the first crab stage, and since this stage of the only other *Pinnotheres* in which it is known, *viz. P. taylori,* described by Hart (1935), closely resemble the other two in having long, plumose swimming hairs on the third and fourth pairs of pereiopods, there is every reason to expect this to be the invasive stage in all the species. Consequently we do not agree with Atkins (1954, 1955), who suggested that *P. pinnotheres,* but not *P. pisum,* possibly invades its host in the megalopa stage. Atkins draws attention to a paper by Wells (1940) who found the megalopa of two different species of *Pinnixa* inside their bivalve hosts. This genus differs, however, from *Pinnotheres* in many respects as is also indicated by its systematic position in a different subfamily, and the only known first crab stage of this genus, *viz.* that of *P. sayana,* described by Faxon (1879), apparently does not possess plumose swimming hairs. It seems more significant that Wells never found the megalopa of *Fabia subquadrata,* a species closely related to *Pinnotheres,* although he examined a large number of the bivalves in which this species commonly occurs.

From the above account it follows that neither the male *Pinnotheres* nor the immature females are free-living as so often stated in the literature (Rathbun, 1918; Orton, 1921; Berner, 1952; and others). The free-swimming period of the male during the copulatory period may vary in length for the different species, but it is, nevertheless, only a phase in the otherwise commensal or parasitic life of the crab.

In some species, however, the young invasive stage crabs may invade a host in which they do not occur as adults, and in these cases both sexes migrate from the initial to the final host upon reaching the hard stage. This has been shown to be the case in *P. pisum,* which apparently exhibits a regular host change, the invasive and pre-hard stages having been found only in the clam, *Spisula solida.* Most probably these stages will also be found in other bivalves of the same type, but only the hard-stage crabs may be found in the initial as well as the final host species. Other *Pinnotheres* with a host change should probably be looked for among those species of which free-swimming hard stage females have frequently been taken.

Thus, the specialized hard stage, which primarily has evolved to serve the purpose of uniting the two sexes for copulatory activities, may also serve another important function. Under certain conditions it is also conceivable that females of species which do not normally change host may do so, but in such cases no change of host species needs to be involved. If a number of female oyster crabs invade the same host during the same invasive season, only one will reach the mature stage within that particular oyster (Stauber, 1945). Excessive females therefore either perish or migrate to other oysters. As discussed earlier, mortalities involving all crabs but one usually take place in spat, but this is not the case in yearlings and older oysters. In fact, Stauber (1945, and unpublished data) found a very large number of older oysters containing 10 or more immature, mostly hard stage crabs in early winter. It therefore seems very possible that the excessive females may survive the winter within the host and migrate to other, non-infested oysters the following spring.

The puzzling occurrence of soft-shelled male *P. ostreum* as large or larger than hard-stage males has been touched upon earlier. Since only few soft-shelled males were found after the mass development of pre-hard crabs into the hard stage in June and the first half of July in 1956 (Fig. 5), we became at first convinced that they were normal pre-hard crabs delayed in their development, especially since the evidence derived from other field data, as well as from the moulting experiments, supported this conclusion. However, while all these data certainly show that hard-stage males do not normally moult into soft-shelled crabs, the recent studies on *P. pisum* indicate that a specimen may now and then do so. If, namely, the preliminary results are correct with regard to the mentioned regular change of host, pre-hard crabs should not occur in host species that act as host for the adult crab, yet soft-shelled males have been taken from *Mytilus edulis* by several authors, including Atkins (1933). It is significant, however, that only very few have been taken while the hard stage has been taken in large numbers. Nevertheless, unless the crab occasionally enters the normal final host already while in the invasive stage, these soft-shelled males must derive from hard-stage crabs, and there are reasons to believe that the latter hypothesis is true. The conspicuously large soft-shelled males of *P. ostreum* usually occur in late summer, and although Atkins (1933) made collections throughout the year, she found two of her soft-shelled specimens on June 6th and the remaining six in early August, *i.e.* in both species they seem to occur mainly after all the pre-hard males should have developed into the hard stage. This poses the still unanswered question as to what may cause such abnormal moultings. Atkins (1933), who did not know about the existence of the soft-shelled pre-hard stages, also wondered about this. She dispelled the statement of Mercier and Poisson (1929) that they were caused by a parasitic isopod, and she found no other parasites in the eight specimens examined by her. As Atkins is a known authority on parasites of *Pinnotheres* it is most unlikely that any were overlooked. One possibility which deserves attention is whether a tendency toward protandric hermaphroditism might be involved since it seems to be the only other factor that could explain the moulting of hard-stage males into a soft-shelled stage very much like the female second stage. An examination of the gonads of freshly caught specimens might help to solve the problem.

Orton (1921) discovered that the female *P. pisum* copulates precociously, and this has been confirmed for *P. ostreum*. However, since the two sexes develop through exactly the same stages until and including the (hard) stage in which the copulation takes place, this is evidently also true for the male. In other words, post-hard males, equivalent to the existing female stages, probably existed somewhere in the line of evolution. When they were no longer needed for the survival of the species they disappeared. There can be little doubt that these phenomena are the results of evolutionary adaptations to the commensal or parasitic life of the genus. The ability of copulating in the hard stage, together with the added adaptations of this stage for swimming and entering a host, makes it possible for the two sexes to inhabit different host specimens except when mating takes place. And due to the small size and shortened life of the male, the available space and food in the host population is most effectively utilized by the crab population, *viz.* for the vital production of eggs, the number of which is highly dependent on the number and size of the crabs producing them.

In summary it may be said that *Pinnotheres* seems more adapted to its mode of life than hitherto believed, and the genus must have a long evolutionary history behind it. The post-planktonic developmental cycle with its specialized stages, as well as the reproductive biology, is unique and quite unlike anything known from free-living crabs.

The very considerable differences which exist in the development, morphology and biology of pinnotherid crabs make it difficult to judge on which points some of the present findings may also apply to certain other genera. Even within the genus *Pinnotheres* itself there are notable differences, especially with regard to the larvae, a fact which has made Lebour (1928) and others wonder whether they could all belong to the same genus. In short, both from a systematic and biological point of view the pea crabs offer a promising field of research.

Summary

1. It is shown that *Pinnotheres ostreum* invades its host, *Crassostrea virginica* in the first crab stage and not in the hard-shelled stage as hitherto believed.

2. The finding of the first crab stage both in plankton samples and inside oysters marks the first find in nature of this stage for any pinnotherid crab.

3. A preliminary description is given of the following *pre-hard* stages, which were the last unknown stages in the developmental cycle of *P. ostreum.* Both sexes of these stages were found only in the oyster and are never free-living. A full life-history of a pinnotherid crab is now known for the first time.

4. Invasion of the oyster in Delaware Bay takes place in late summer at a time when most of the oyster spat have grown sufficiently large to harbor one or more crabs.

5. More crabs invade spat than yearlings and older oysters, but the survival rate is higher for crabs invading the latter groups of oysters.

6. The growth rate of the crab from the invasive to the mature stage is shown to be positively correlated with the growth rate of the host.

7. Development of the crab is not retarded in slow-growing oysters to the same extent as the rate of growth. This results in a considerable size variation of female crabs just moulted into the mature stage, *viz.* from 4.4 to about 9.0 mm. in carapace width.

8. The *hard stage,* hitherto believed to be the invasive stage, is shown to be a specialized stage which primarily serves the purpose of uniting the two sexes for copulatory purposes. The males leave their hosts in this stage to search for females in other oysters, but this free-swimming period is only a phase in the otherwise parasitic life of the crab.

9. It is shown that males do not develop beyond the hard stage. They disappear shortly after copulation with one or more females, which usually takes place in late

June and in July. In contrast to the females they therefore only become one year old or less.

10. Females become ovigerous in their first summer but do not reach maximum size before their second summer. At least some of them become three years old.

11. Ovigerous crabs are found in Delaware Bay from early June to middle October with a distinct maximum occurring between late July and late August. The older crabs deposit eggs before the one-year-old crabs. The eggs are carried from three to five weeks, and the length of the larval period is three to four weeks.

12. The possible influence of *P. ostreum* on the growth and reproduction of the host is discussed. It is believed that the crab exerts no (discernible) influence in its first year but that it probably does in many cases in its second (and third) year.

LITERATURE CITED

Amemiya, I., 1929. On the sex change in the Japanese common oyster *Ostrea gigas* Thunberg. *Proc. Imp. Acad. Tokio,* **5**: 284–286.

Amemiya, I., 1935. Effect of gill excision upon the sexual differentiation of the oyster *Ostrea gigas* Thunberg. *Rep. Jap. Ass. Adv. Sci.,* **10**: 1025–1028. Abstr. in *Jap. J. Zool.,* **6**: (84).

Atkins, D., 1926. The moulting stages of the pea-crab (*Pinnotheres pisum*). *J. Mar. Biol. Assoc.,* **14**: 475–493.

Atkins, D., 1933. *Pinnotherion vermiforme* Giard and Bonnier, an Entoniscid infecting *Pinnotheres pisum. Proc. Zool. Soc. London,* 1933; 319–363, 6 pl.

Atkins, D., 1954. Leg disposition in the Brachyuran megalopa when swimming. *J. Mar. Biol. Assoc.,* **33**: 627-636.

Atkins, D., 1955. The post-embryonic development of British *Pinnotheres* (Crustacea). *Proc. Zool. Soc. London,* **124**: 687–715.

Awati, P. R., and H. S. Rai, 1931. *Ostrea cucullata. Indian Zool. Memoirs,* **3**: 107 pp.

Berner, L., 1952. Biologie de *Pinnotheres pisum* Penn. (Décapode, Brachyoure). *Bull. Soc. Zool. France,* **77**: 344–349.

Bürger, O., 1895. Ein Beitrag zur Kenntnis der Pinnotherinen. *Zool. Jahrb. Abt. Syst.,* **8**: 361–390.

Coe, W. R., 1934. Alternation of sexuality in oysters. *Amer. Nat.,* **68**: 1–16.

Coupin, H., 1894. Sur l'alimentation de deux commensaux (*Nereilepas* et *Pinnotheres*). *C. R. Acad. Sci. Paris,* **119**: 540–543.

Darbishire, A. D., 1900. Investigations made at the Marine Biological Laboratory, Plymouth. Rep. Brit. Assoc. Adv. Sci., Bradford; 399.

Egami, N., 1953. Studies on sexuality in the Japanese oyster, *Ostrea gigas.* VII. Effects of gill removal on growth and sexuality. *Annot. Zool. Japon.,* **26**: 145–150.

Enders, H. E., 1905. Notes on the commensals found in the tubes of *Chaetopterus pergamentaceus. Amer. Nat.,* **39**: 37–40.

Faxon, W., 1879. On some young stages in the development of *Hippa, Porcellana,* and *Pinnixa. Bull. Mus. Comp. Zool., Harvard,* **5**: 253–268.

Freame, M. E., 1943. Crab-mussel association. *Victorian Naturalist,* **59**: 156.

Gerstaecker, A., and A. E. Ortmann, 1901. Malacostraca. In: Bronn's Klassen und Ordnungen des Thier-Reichs, 5: Pt. I, 1319 pp.

Hart, J. F. L., 1935. The larval development of British Columbia Brachyura. I. Xanthidae, Pinnotheridae (in part) and Grapsidae. *Canad. J. Res.,* **12**: 411–432.

Hornell, J., and T. Southwell, 1909. Description of a new species of *Pinnotheres* from *Placuna placenta,* with a note on the genus. Report to the government of Baroda on the marine zoology of Okhamandal in Kattiawar, Pt. I. 99–103. London.

Hyman, O. W., 1924. Studies on larvae of crabs of the family *Pinnotheridae. Proc. U. S. Nat. Mus.,* **64**: 1–9.

Korringa, P., 1952. Recent advances in oyster biology. *Quart. Rev. Biol.,* **27**: 266–308.

Lebour, M. V., 1928. Studies on the Plymouth Brachyura. II. The larval stages of *Ebalia* and *Pinnotheres. J. Mar. Biol. Assoc.,* **15**: 109–123.

MacGinitie, G. E., and N. MacGinitie, 1949. Natural History of Marine Animals. McGraw-Hill Book Co., New York, 473 pp.

Mercier, L., and R. Poisson, 1929. Alteration de certaines caractères sexuels secondaires du mâle de *Pinnotheres pisun* L. parasité par un Entoniscien (*Pinnotherion vermiforme* Giard et Bonnier). *Bull. Soc. Zool. France*, **54**: 301–304.

Miyake, S., 1935. Note on the zoeal stages of *Pinnotheres latissimus*. *Bull. Sci. Facult. Terkultura, Kiusu Imp. Univ.*, **6**: 192–201.

Orton, J. H., 1921. The mode of feeding and sex phenomena in the pea-crab (*Pinnotheres pisum*). *Nature*, **106**: 533–534.

Overcash, A. E., 1946. The use of measurement to determine the condition of oysters in Virginia. Master's thesis, College of William and Mary, 31 pp. Unpublished. (Cited from Sandoz and Hopkins, 1947.)

Rathbun, M. J., 1918. The Grapsoid crabs of America. *Bull. U. S. Nat. Mus.*, No. 97, 461 pp. 161 pl.

Ryan, E. P., 1956. Observations on the life histories and the distribution of the Xanthidae (mud crabs) of Chesapeake Bay. *Amer. Midl. Nat.*, **56**: 138–162.

Sakai, T., 1939. Studies on the Crabs of Japan. IV. Brachygnatha, Brachyrhynca. 365–741. Tokyo.

Sandoz, M., and S. H. Hopkins, 1947. Early life history of the oyster crab, *Pinnotheres ostreum* (Say). *Biol. Bull.*, **93**: 250–258.

Semper, K. G., 1881. Animal Life as Affected by the Natural Conditions of Existence. Appleton, New York, 472 pp.

Stauber, L. A., 1945. *Pinnotheres ostreum*, parasitic on the American oyster, *Ostrea* (*Gryphaea*) *virginica*. *Biol. Bull.*, **88**: 269–291.

Tesch, J. J., 1918. The Decapoda Brachyura of the Siboga Expedition. II. Goneplacidae and Pinnotheridae. Siboga-Expeditie, 39 c¹: 295 pp. 18 pl.

Thompson, J. V., 1835. Memoirs on the metamorphosis and natural history of the *Pinnotheres* or pea-crabs. *Entom. Mag.*, **3**: 85–90. London.

Tu, Tseng-Jui, 1938. Über einige in Polychaetenröhren und Muscheln gefundene Dekapoden an der Küste von Schantung. *Zool. Anz.*, **122**: 177–186.

Verrill, A. E., and S. I. Smith, 1874. Report upon the invertebrate animals of Vineyard Sound and adjacent waters, etc. Rept. U. S. Comm. Fish and Fisheries, 1871–1872, 478 pp. 38 pl.

Wells, W. W., 1928. Pinnotheridae of Puget Sound. *Publ. P. S. Biol. Sta.*, **6**: 283–314.

Wells, W. W., 1940. Ecological studies on the pinnotherid crabs of Puget Sound. *Univ. Wash. Publ. Oceanog.*, **2**: 19–50.

Welsh, J. H., 1932. Temperature and light as factors influencing the rate of swimming of larvae of the mussel crab, *Pinnotheres maculatus* Say. *Biol. Bull.*, **63**: 310–326.

SOME EFFECTS OF OXYGEN UPON THE WHITE PUPAE OF HABROBRACON [1]

A. M. CLARK AND M. J. PAPA

Department of Biological Sciences, University of Delaware, Newark, Delaware

Various investigations on a wide range of organisms have shown that high pressures of oxygen may have deleterious effects (Stadie, Riggs and Haugaard, 1944; Bean, 1945). Although injury from oxygen has been reported for insects the data have been rather meager. In 1878, Paul Bert in his studies on oxygen poisoning for a wide range of organisms reported that beetles are killed by high pressures of oxygen (Bean, 1945). A toxic effect from oxygen has been shown by Williams and Beecher (1944) for *Drosophila azteca* adults. Glass and Plaine (1952) reported a slight lag in development for *Drosophila melanogaster* exposed as embryos. Goldsmith and Schneiderman (1956) reported that various post-embryonic stages of *Mormoniella vitripennis* are sensitive to oxygen. Injurious effects from exposure to oxygen have been shown for *Habrobracon juglandis* where an arrestment of development, arrestment of pigmentation and a decrease in oxygen consumption occurs (Clark and Herr, 1954). The marked sensitivity of *Habrobracon* during certain stages of development indicated that this may be a good organism on which to study the mechanism of oxygen poisoning. In the present paper there are presented (1) data on the sensitivity of white pupae to oxygen, (2) data on the modification of oxygen-sensitivity by temperature.

Materials and Methods

The methods of rearing and of experimentation on *Habrobracon* have appeared in previous publications (Clark and Mitchell, 1951). Virgin females from Stock No. 33 were allowed to lay eggs during four hour periods. These eggs are haploid and accordingly develop into males. The cultures were allowed to develop for six days (approximately 144 hours). At this age all of the wasps are in the white pupal stage. Groups of pupae were placed into plastic chambers of about 100 $cm.^3$ in volume. The chambers were flushed for one minute with 100 per cent oxygen delivered from a commercial cylinder and then exposed to oxygen at the desired pressure for one additional minute. The pupae were then removed from the plastic chamber into an air environment and observed for effects upon development and upon oxygen consumption. The eclosion ratio, the incidence of adults that emerge from cocoons, was used as a measure of survival. Groups of pupae that were exposed to 2 atmospheres of nitrogen showed no deleterious effects. This indicates that the injury reported here is not due to pressure.

Previous work has shown that the larval stages are resistant and the early pupal stages are quite sensitive to oxygen (Clark and Herr, 1954). White pupae (6-day

---

[1] Research carried out under AEC contract AT(30–1)–1752 between the University of Delaware and the U. S. Atomic Energy Commission.

cultures) appear to be most sensitive and were, therefore, selected for further experimentation. Pigmented pupae (7-day cultures), however, yield results comparable to those reported here for white pupae. The cultures were culled before use in order to remove individuals that were not in this stage, that had died, or that were too small. *Habrobracon* has a life cycle of 9 days at 30° C.

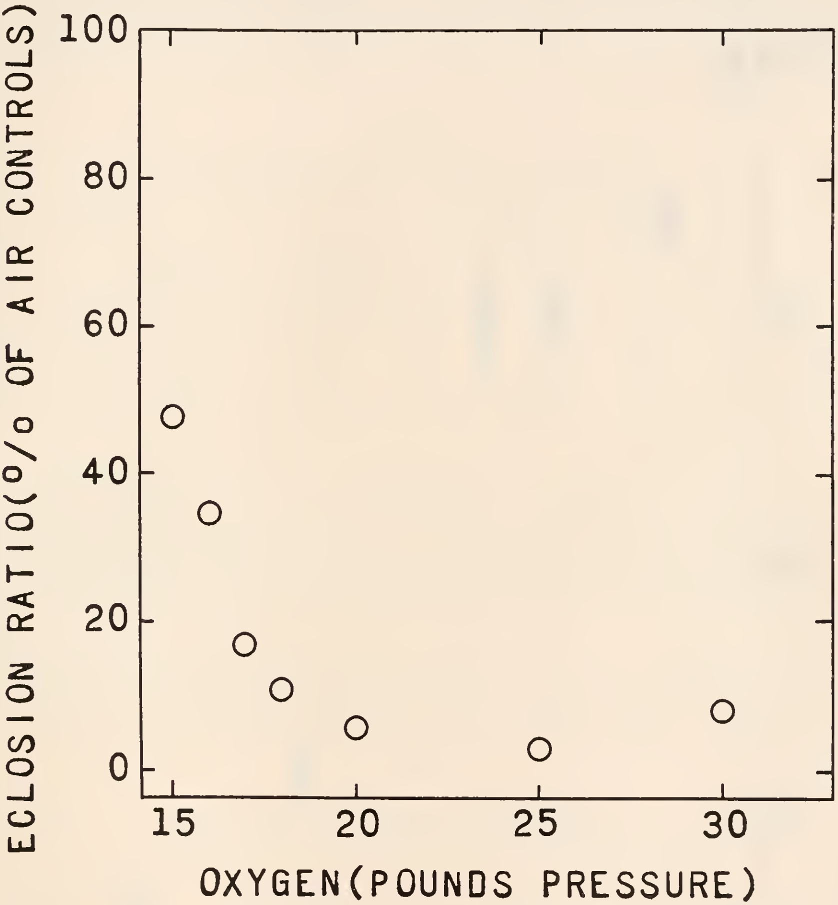


FIGURE 1. Eclosion ratios for wasps that were exposed to 100 per cent oxygen (15 to 30 pounds for one minute).

RESULTS

Groups of pupae were exposed to oxygen at pressures from 15 pounds to 30 pounds. A marked effect on the ability of the pupae to develop to the adult stage and to emerge from cocoons was observed. With increased pressures of oxygen from air to 20 pounds oxygen there is a decided decrease in the percentage of pupae that develop to the adult stage. After exposure to 20 pounds of oxygen only 5 per cent of the pupae develop to the adult stage (Fig. 1). The slight increase in eclosion for groups of pupae exposed to 30 pounds of oxygen is spurious since most of the experiments at this dosage of oxygen yield zero eclosion. In fact, exposure of white pupae to 30 pounds of oxygen for only five seconds will arrest their development. Of 49 white pupae that were treated with 30 pounds of oxygen for 5 seconds, none developed to the adult stage.

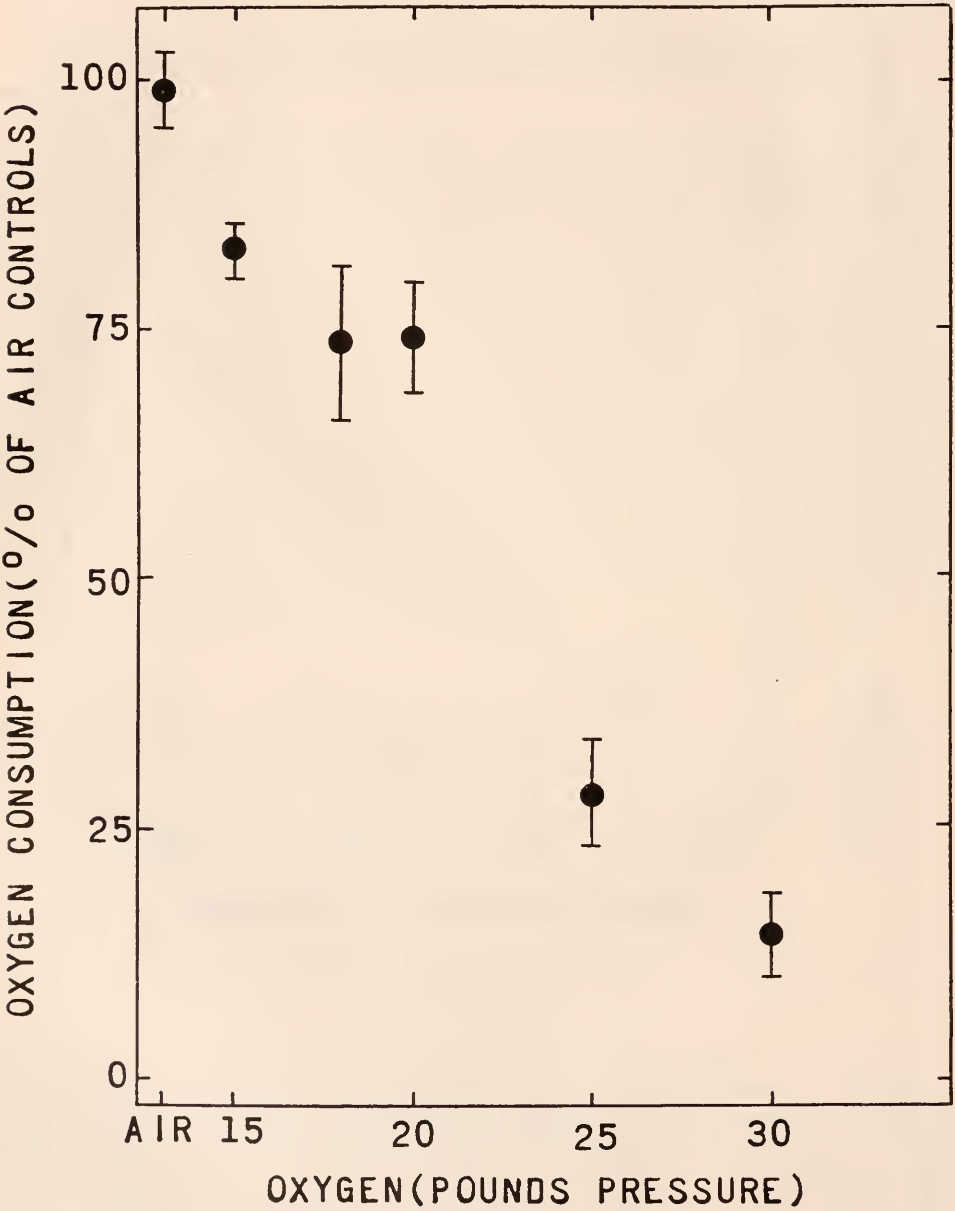


Figure 2. Oxygen consumption for white pupae that were exposed to 100 per cent oxygen (15 to 30 pounds for one minute).

White pupae treated with thirty pounds of oxygen are arrested at this stage of development. They remain as white pupae for about a week and after this time may become somewhat pigmented. They appear to be alive for at least two weeks as indicated by the lack of discoloration or the absence of drying-out of the pupae. Further, such pupae showed the same magnitude of oxygen consumption after two days as after one hour. White pupae exposed to 15 pounds of oxygen become pigmented before they are arrested in development while white pupae treated with 30 pounds of oxygen are arrested immediately as white pupae. Within these extremes the amount of delay in pigmentation is influenced by the pressure of oxygen that is applied.

TABLE I

Respiratory quotients of white pupae exposed to oxygen

Treatment		Expressed as ul/25 pupae/hr.			
Gas	Pressure (pounds)	No. of experiments	O_2 consumed	CO_2 liberated	R. Q.
Air	15	6	40	27	.67
Oxygen	15	6	29	18	.61
Oxygen	20	6	15	10	.70
Oxygen	25	6	10	8	.77

Groups of pupae (25 pupae/group) were treated with oxygen and then measured for oxygen consumption with a Warburg respirometer. The amount of oxygen utilized by the pupae was found to decrease as the oxygen pressure was increased (Fig. 2).

Comparison of the per cent of eclosion (Fig. 1) and the oxygen consumption (Fig. 2) shows that the degree of decrease is not of the same magnitude in each. The eclosion ratio drops off faster than the rate of oxygen uptake. After exposure to an oxygen pressure of 20 pounds the eclosion falls to about 5 per cent of the air controls while for the oxygen uptake it falls to about 75 per cent of the air controls. Thus, the decrease in the eclosion percentage is most marked in the oxygen dosage range from air to 20 pounds of oxygen, while the greatest decrease in oxygen consumption does not occur until after treatment within the dosage range of 20 to 30 pounds of oxygen. The data indicate that there is no difference in oxygen uptake between pupae treated with 18 pounds or with 20 pounds of oxygen (Fig. 2).

TABLE II

Oxygen uptake for white pupae exposed to oxygen
(measured 1 and 24 hours after treatment)

Treatment		ul O_2/25 pupae/hr.	
Gas	Pressure (pounds)	1 hour	24 hours
Air	15	38	32
O_2	15	28	26
O_2	20	11	12
O_2	25	8	8

The respiratory quotient (R. Q.) was determined after exposure to various pres-

TABLE III

Eclosion ratios of white pupae exposed to oxygen at 10° C. and 26° C.

Treatment		Eclosion ratio	
Gas	Pressure (pounds)	26° C.	10° C.
Air	15	74–82	41–47
O_2	15	12–58	48–57
O_2	18	7–41	47–55
O_2	20	5–101	79–93
O_2	25	0–36	34–50

sures of oxygen (Table I). In these experiments groups of pupae were measured for oxygen consumption for three hours, after which the KOH was removed from the center well and the amount of carbon dioxide liberated was determined. With increasing pressures of oxygen there is a decrease both in the oxygen consumption and in the liberation of carbon dioxide. There is no change in the R. Q. with increased oxygen pressure (Table I).

It seemed of interest to inquire whether there was any recovery of the ability to consume oxygen following treatment with oxygen. In order to test this, pupae whose oxygen consumption had been measured within one hour after treatment with oxygen pressures from 15 to 25 pounds were kept in an incubator for 24 hours and then re-measured for oxygen consumption. These data appear in Table II and show that there is no change in oxygen uptake after 24 hours. Experiments not reported here have shown that the oxygen consumption does not increase after two days. Thus, the decrease in oxygen consumption following oxygen treatment is irreversible.

TABLE IV

Oxygen uptake of white pupae exposed to oxygen at 10° C. and 26° C.

Treatment		ul O_2/25 pupae/hr.	
Gas	Pressure (pounds)	26° C.	10° C.
Air	15	44	46
O_2	25	21	47

Groups of white pupae were placed into a refrigerator at 10° C. for ½ hour or kept at room temperature (26° C.) for the same length of time. They were then exposed immediately to oxygen of known pressure and placed into the incubator (30° C.) to observe for developmental effects. Pupae treated when cold were much more resistant to oxygen than were the pupae that were treated when warm (Table III). For example, of 50 pupae that were treated with 25 pounds of oxy-

TABLE V

Eclosion ratios of white pupae treated with oxygen before and after exposure to 10° C. and 26° C.

	Eclosion ratio Oxygen pressure (pounds)		
Treatment	15	20	25
10° C., then O_2, then 10° C.	91–106	62–72	26–43
26° C., then O_2, then 10° C.	34–90	1–58	0–30
10° C., then O_2, then 26° C.	84–96	34–44	33–41
26° C., then O_2, then 26° C.	35–76	3–42	0–35

gen after exposure to 10° C., 35 developed to the adult stage and emerged from their cocoons while of 36 pupae treated in the same manner after exposure to 26° C., none developed to the adult stage and eclosed. Other groups of pupae were treated in the same manner and measured for oxygen uptake. Pupae exposed to 25 pounds of oxygen after cold exposure consumed as much oxygen as the controls while pupae treated after exposure to warm temperature showed a marked decrease in oxygen consumption (Table IV). These data on oxygen consumption are in agreement with the data on eclosion (Table III).

Since temperature has an effect on the sensitivity of pupae to oxygen, the possibility that this oxygen-sensitivity could be modified by exposure to different temperatures after oxygen treatment was considered. Groups of white pupae were placed either at 10° C. or 26° C. for ½ hour, then exposed to oxygen of known pressure and then placed at 10° C. or at 26° C. for one hour (Table V). Eclosion ratios were obtained from the pupae so treated and showed that the post-treatment with temperature had no effect upon recovery. Thus, the temperature at the time of treatment with oxygen modified the oxygen-sensitivity. Whether longer periods of post-treatment with temperatures of 10° C. would be effective has not been tried. The metabolic state of the organism at the time of treatment seems, therefore, to determine the extent of its sensitivity.

DISCUSSION

Habrobracon white pupae when exposed to oxygen show an immediate and marked decrease in oxygen consumption and, subsequently, an arrestment of development and of pigmentation. The magnitude of these effects can be correlated to a certain degree with the dosage of oxygen that is applied to these organisms.

It seems clear that the arrestment of pigmentation is due to the lack of sufficient oxygen in the tissues to allow for the enzymatic oxidation of tyrosine to melanin. This seems to be indicated by the following observations. The steep drop in the pigment-forming ability occurs after those dosages of oxygen where a marked decrease in oxygen consumption occurs (between 20–30 pounds pressure, Figure 2). At doses of less than 20 pounds of oxygen, there is relatively little decrease in pigmentation and in oxygen consumption. The arrestment of pigmentation in *Habrobracon* white pupae can be brought about also by exposure of the pupae to lowered concentrations of oxygen. There is no recovery in the rate of oxygen consumption for pupae whose oxygen consumption has been lowered by exposure to oxygen. It is generally realized that an arrestment of pigmentation may be caused by exposure of insects to environments with less oxygen tension. The fact that arrestment of pigmentation may be caused by increased oxygen pressures was reported by Linden in 1906 (Sussman, 1949).

The events that are responsible for the arrestment of development may be different from those responsible for the arrestment of pigmentation since pupae that are exposed to 15 pounds of oxygen show an arrested development but exhibit no delay in the acquiring of pigment. It seems difficult to relate this arrested development to a decrease in available oxygen since the incidence of pupae that develop to the adult stage after exposure to 20 pounds of oxygen is low (5 per cent of controls) while their rate of oxygen consumption is relatively high (75 per cent of controls). It is possible that the arrested development may be due to the inactivation of a substance that has some control over development or to an increased concentration of some toxic materials.

Various authors (see Bean, 1945) have suggested that the primary effect of exposure to oxygen gas is the inactivation of oxidative enzymes with a resultant generalized tissue anoxia. The fact that there is an immediate decrease in oxygen consumption for *Habrobracon* pupae indicates that this hypothesis may be valid. Studies on the oxygen uptake and enzyme activity of tissue homogenates, at present in progress, are needed to show this. To date, however, no decrease in oxygen consumption or in succinic dehydrogenase activity of homogenates from oxygen-treated pupae has been observed. Extensive experiments bearing on this hypothesis of tissue anoxia have been carried out by Stadie, Riggs and Haugaard (1944) with negative results. They found no immediate reduction in oxygen uptake in tissues from rats that had been killed by 7 atmospheres of oxygen. They assume, therefore, that generalized tissue anoxia is not the cause of acute oxygen poisoning. Despite this, it is not possible to eliminate the possibility that localized tissue anoxia may occur.

All stages of development in *Habrobracon* are not equally sensitive to the injurious effects of oxygen (Clark and Herr, 1954). The larval and prepupal stages are not affected by 30 pounds of oxygen, while almost all of the pupae are injured. The reason for this difference in stage-sensitivity is not known at present. It seems clear, however, that it is not due simply to a difference in the rate of metabolism. Based upon oxygen consumption studies one can show that the oxygen-resistant larvae are more active than are the oxygen-sensitive pupae. In the present paper, however, experiments have been given that show that pupae that have been made less active by exposure to a temperature of 10° C. are more resistant to the toxic effects of oxygen than are pupae that were kept at 26° C. immediately before treatment. It seems that some qualitative difference in the metabolism of larvae and pupae exists that can be related to this difference in sensitivity. Our primary aim, then, is to determine the nature of these differences during development.

The marked and immediate decrease in oxygen consumption for *Habrobracon* pupae and the absence of a compensating recovery is surprising. In the wasp *Mormoniella vitripennis,* exposure of black pupae to 5 atmospheres of oxygen for from 4 to 6 hours prevented 50 per cent from emerging but their oxygen uptake was unimpaired (Goldsmith and Schneiderman, 1956). We have treated pupae of other insect species with 2 atmospheres of oxygen under conditions comparable to those that we used for *Habrobracon* but no obvious effects on oxygen uptake or on development have been observed. The species tested were *Drosophila melanogaster, Musca domestica, Ephestia kuhniella* and *Polistes* sp. It is hard to imagine that other species of insects do not exist that exhibit strong oxygen-sensitivity and, therefore, our search for other insects in this category continues.

The authors wish to express their appreciation to Dr. James B. Krause and to Dr. Richard Darsie for helpful suggestions concerning the manuscript.

Summary

1. *Habrobracon* were exposed as white pupae to oxygen and studied for effects upon development, oxygen consumption and pigmentation.

2. A marked decrease in the incidence of pupae that complete development occurs after exposure to oxygen within the range from air to 20 pounds. The greatest decrease in the rate of oxygen uptake and pigmentation occurs after exposure within the range from 20 to 30 pounds.

3. The decrease in oxygen uptake following treatment is immediate. No subsequent recovery of oxygen uptake was observed 24 hours after treatment.

4. There is no modification of the respiratory quotient following treatment with oxygen. With increasing pressures of oxygen both the oxygen consumption and carbon dioxide liberation decrease at the same rate.

5. The sensitivity of white pupae to oxygen is modified by temperature. Pupae treated when cold are more resistant than pupae treated when warm. Thus, lowering the metabolic state of the pupae increases their resistance to oxygen.

6. The inability of the oxygen-treated pupae to acquire pigmentation has been explained on the basis of insufficient oxygen to allow for the oxidation of tyrosine to melanin. The effect of the oxygen treatment upon oxygen consumption and on development is unexplained and at present obscure.

LITERATURE CITED

Bean, J. W., 1945. Effects of oxygen at increased pressure. *Physiol. Rev.*, **25**: 1–147.

Clark, A. M., and E. B. Herr, Jr., 1954. The sensitivity of developing *Habrobracon* to oxygen. *Biol. Bull.*, **107**: 329–334.

Clark, A. M., and C. J. Mitchell, 1951. Radiosensitivity of haploid and diploid *Habrobracon* during pupal development. *J. Exp. Zool.*, **17**: 489–498.

Glass, B., and H. L. Plaine, 1952. The role of oxygen concentration in determining the effectiveness of X-rays on the action of a specific gene in *Drosophila melanogaster*. *Proc. Nat. Acad. Sci.*, **38**: 697–705.

Goldsmith, M. H., and H. A. Schneiderman, 1956. Oxygen poisoning in an insect. *Anat. Rec.*, **125**: 560.

Stadie, W. C., B. C. Riggs and N. Haugaard, 1944. Oxygen poisoning. *Amer. J. Med. Sci.*, **207**: 84–114.

Sussman, A. S., 1949. The functions of tyrosinase in insects. *Quart. Rev. Biol.*, **24**: 328–341.

Williams, C. M., and H. K. Beecher, 1944. Sensitivity of *Drosophila* to poisoning by oxygen. *Amer. J. Physiol.*, **140**: 566–573.

ON DEVELOPMENT OF EARLY STAGES OF UROSALPINX CINEREA (SAY) AT CONSTANT TEMPERATURES AND THEIR TOLERANCE TO LOW TEMPERATURES

ANTHONY E. GANAROS

U. S. Fish and Wildlife Service, Milford, Conn.

One of the most destructive predators of young oysters in Long Island Sound is the oyster drill, *Urosalpinx cinerea* (Say). However, our knowledge of its early development and the tolerance of its egg cases to winter temperatures, when deposited late in the season, remains incomplete. Carriker's (1955) comprehensive review of the literature on oyster drills clearly shows that most workers, while mentioning the time needed for ova to develop into young conchs, neglect to give the temperature ranges at which development occurs. Among the few who offer information on this subject, Haskin (1935) states that within the temperature range of 23.3° to 29.1° C., 18 to 25 days are needed for the first protoconch to hatch. Federighi (1931) reports that within a range of 18.0° to 32.0° C., it takes approximately 40 days to complete the development. Stauber's field data (Carriker, 1955) indicate that within the temperature range of 15.0° to 25.0° C., from 45 to 78 days are required for drill eggs to develop. Cole (1942) reports 27 to 32 days at 22.6° C., and 44 to 55 days at 18.3° C.

As can be seen from the above references, the information is insufficient to form precise conclusions. We, therefore, devised experiments to determine more accurately the rate of early development of drills at several constant temperatures, which may be encountered within the temperature range of Long Island Sound or adjacent waters.

It was also considered of theoretical interest and practical importance to learn the fate of the eggs deposited so late in the fall that they cannot complete development. Such egg cases, collected during the winter from subtidal and intertidal zones, frequently contain live ova and veligers. Yet, no systematic observations on whether these eggs and embryos can survive the winter and be released in the spring have ever been made. If these embryos could develop, they would have an early start, thus adding to the destructive potential of the next year-class of drills.

METHODS

Egg cases were obtained from drills maintained in the laboratory at 20.0° C. Preliminary experiments were made with egg cases scraped from the shells of the oysters used to feed the drills and from the glass sides of the aquarium. They were examined under a dissecting microscope and only those that had non-segmented ova, a soft pliable outer membrane, and a translucent bluish-white appearance were selected. It was estimated that such egg cases had been deposited within three days. Later, clusters of young oysters were placed in the aquarium overnight, and the egg cases deposited on them were used in the experiments. Thus, the age of these egg cases was known to be not more than 16 hours.

To determine the rate of development of ova at different constant temperatures, 20 egg cases were placed in perforated, transparent, plastic containers weighted with lead, which were then put in trays. Each tray was supplied with a constant flow of sea water at a salinity of about 25‰, and the temperature of the water was maintained at 7.5°, 10.0°, 15.0°, 20.0°, 25.0° and 30.0° C., controlled to within ± 1.0° C. (Loosanoff, 1949).

In addition to conducting experiments at the above constant temperatures, egg cases were also kept at chilling winter temperatures just above freezing, and others were exposed to sub-freezing temperatures in and out of sea water. For observations on the effects of the chilling temperature, 26 egg cases, dredged from New Haven Harbor on December 5, 1955, were suspended in outdoor tidal tanks and systematically examined until March 22, 1956. Egg cases were also taken in winter from an aquarium kept at 20.0° C. and gradually conditioned to the Harbor water temperature which, at that time, was approximately 1.3° C. On January 7, 1956, they were placed in the Harbor and kept there until July 20, 1956.

To expose the egg cases to sub-freezing temperatures, they were placed in the freezing compartment of a refrigerator. In the first experiment the temperature of the compartment was − 12.0° C. (± 1.0° C.), and in the second, − 16.0° C. (± 1.0° C.). In each experiment the egg cases, with ova and veligers, were preconditioned in sea water to 3.0° C. Altogether nine plastic boxes, each containing 20 egg cases, were used. Six containers were without water and three contained 100 ml. of sea water each. One container without water was removed at the end of the first half hour and the others at half-hour intervals thereafter, up to three hours. The first group of egg cases kept in sea water was removed after one hour of exposure and the others at hourly intervals. In later, similar experiments, the egg cases were left at both temperatures for two, four and six hours.

DEVELOPMENT AT SIX CONSTANT TEMPERATURES

We determined the number of days required at different temperatures for development of ova (Fig. 1) to the following embryological stages: early larvae (Fig. 2), shelled veliger (Fig. 3), and protoconchs (Fig. 4). The time needed to reach the early veliger stage decreased with increases in temperature from 10.0° to 30.0° C. (Table I). The groups kept at 30.0° and 25.0° C. attained the shelled veliger stage between the fourth and seventh days. However, it required eight additional days for those kept at 20.0° C. to reach the same stage, and even longer for those at 15.0° and 10.0° C.

In spite of the longer time required for ova at 20.0° C. to develop to the shelled veliger stage, they had reached the protoconch stage by the 22nd day, the same as the groups kept at 25.0° and 30.0° C. Nevertheless, the egg cases kept at 30.0° and 25.0° C. started releasing young conchs on the 22nd day, and continued for 16 days for both temperature groups. None of those kept at 20.0° C. were released until the 30th day, and those kept at 15.0° C. were not released until the 56th day. Thus, while there was only eight days' difference in the time required to reach the protoconch stage between the egg cases kept at 20.0° C. and those at 15.0° C., there was 26 days' difference in the time of release of the first young conchs. Moreover, at 20.0° C. the period between the first conch released and the last was only 13 days, while for the 15.0° C. group 22 days were needed.

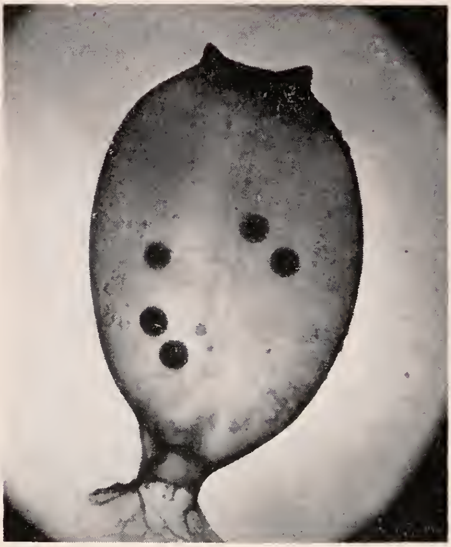


Figure 1. Egg case of *U. cinerea* with five ova. × 10.

At 10.0° C. the ova required 66 days to reach the early veliger stage, and 84 days before the shelled veligers appeared. At 7.5° C. no development occurred during the 54 days. At the end of those periods both the 10.0° C. and 7.5° C. groups were placed in water of 20.0° C. to determine whether they would develop under the new temperature condition, regardless of their previous treatment. Only 65 per cent of the egg cases kept originally at 10.0° C. produced conchs, while no development occurred in the former group at 7.5° C. Since the egg cases at 7.5° C. were apparently adversely affected before being placed at 20.0° C., it may be inferred that temperatures at 7.5° C. and lower not only arrested development, but killed the eggs after a prolonged period of exposure.

Our results indicated that the optimum temperature for drill development was

Table I

Number of days for ova to develop to various stages at constant temperatures and the percentage of egg cases producing young conchs

Stage of development	Temperatures					
	7.5° C.	10.0° C.	15.0° C.	20.0° C.	25.0° C.	30.0° C.
Early veliger	54*	66	10	7	4	2
Shelled veliger	—	84**	25	15	7	7
Protoconch	—	—	30	22	22	22
Release of young conchs	—	—	56–78	30–43	22–38	22–38
Percentage of egg cases producing young conchs	0%	65%	90%	100%	95%	90%

* No development.

** Placed in water of 20.0° C.

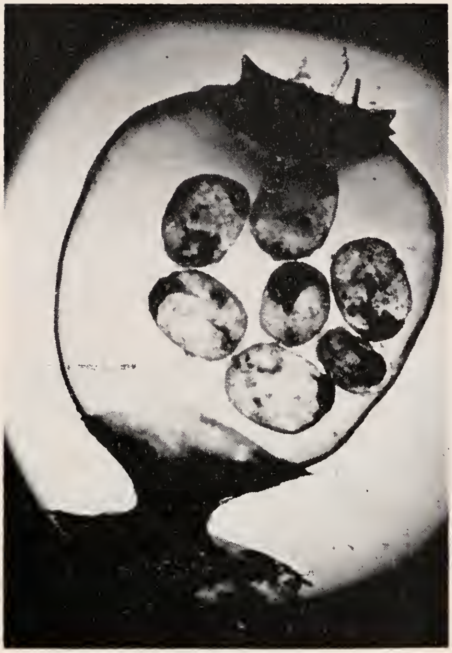


FIGURE 2. Egg case, with outer membrane removed, containing early motile larvae just before a true velum is developed. Larvae at this stage have a gut and can feed on particulate matter by means of currents created by the cilia. × 10.

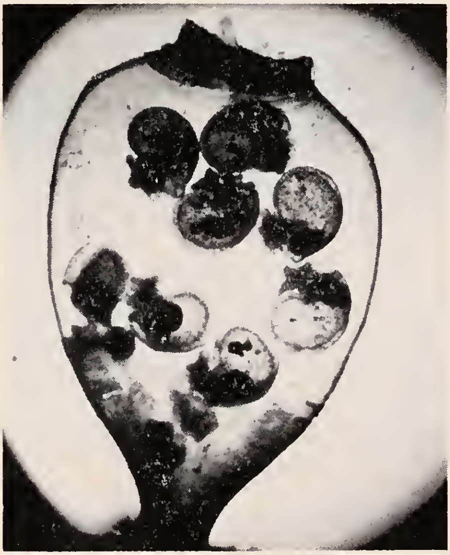


FIGURE 3. Egg case, with outer membrane removed, containing veliger larvae, with foot and velum present, after torsion has occurred. The shell has begun to form along the outer edge of the mantle. × 10.

about 20.0° C. (Table I). However, since the rate of development was faster at 25.0° C. and the difference in the percentage survival of egg cases producing young conchs between 20.0° C. and 25.0° C. was small, it was considered possible that the optimum was above 20.0° C. Therefore, a second experiment was conducted at 20.0° C. and 25.0° C., using 20 egg cases in each temperature group, and the percentage survival of ova determined. At 20.0° C., 139 out of 176 ova, or 78.9 per cent, developed to the protoconch stage, as compared to 96 out of 168 ova, or 56.8 per cent development, at 25.0° C. Thus, although the rate of development was faster at 25.0° C., the optimum temperature, from the standpoint of successful development to the young conch stage, appeared to be about 20.0° C. Moreover, at 20.0° C. the period for protoconch release was shorter than at any other temperature.

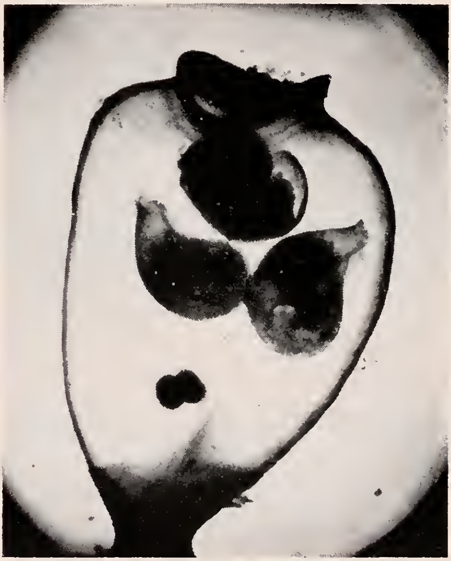


FIGURE 4. Egg case, with outer membrane removed, containing three early protoconch larvae which show partial spiral development and pigmentation. Note also the two undeveloped ova in the same egg case. × 10.

Since the egg cases in the first experiment were deposited within a period of three days, their age alone could not account for the difference of 13 to 22 days between the release of the first and last young conch. The conclusion that slight differences in the age of the cases are not responsible for pronounced differences in the time needed for release of young conchs of the same groups is further substantiated by our second experiment at 15.0° C. in which egg cases collected within a 16-hour period started releasing young conchs at 62 days and continued to do so for 19 days.

TOLERANCE TO LOW TEMPERATURES

To study the tolerance of egg cases to winter temperatures when exposed at low tide and to simulate tide pool conditions, cases containing ova and veligers were placed in water and exposed to air at the same sub-freezing air temperatures.

Because oyster drills in the intertidal zone stay close to the low water level and deposit their egg cases there, the latter are seldom exposed to air for more than an hour or two. Nevertheless, even under these conditions all the eggs would die if exposed to air temperatures around − 15.0° C. However, if the time of exposure at this temperature is reduced to a half hour, approximately 40 per cent may survive. At temperatures around − 12.0° C., five per cent could survive after two hours of exposure.

The survival was greater when the egg cases were protected by water (Table II). At exposure periods of two hours at air temperatures around − 12.0° and − 15.0° C., about 95 per cent of the eggs survived. The chance of survival of eggs in frozen tide pools would be much greater than in air, not only because of the warmer temperatures of the water, but also because desiccation of the egg cases is inhibited. Usually when drill cases were exposed to the sub-freezing air temperatures they were desiccated to such an extent that their walls collapsed. In general, experiments showed that the percentage survival in both air and water decreased with increases in time of exposure and with decreases in temperature (Table II).

TABLE II

Survival of ova and veligers in egg cases of U. cinerea (a) submerged in sea water, and (b) when exposed in air, subjected to two sub-freezing temperatures. Survival is expressed in percentage of egg cases in which shelled veligers developed after returned to 20.0° C. sea water

Air temperature −12.0° C. (±1.0° C.)			Air temperature −16.0° C. (±1.0° C.)		
Length of exposure	Temperature at end of exposure (°C.)	Survival (%)	Length of exposure	Temperature at end of exposure (°C.)	Survival (%)
(a) submerged in sea water					
1 Hr.	− 1.8	100	1 Hr.	− 5.0	90
2 Hrs.	− 2.8	95	2 Hrs.	− 5.0	95
3 Hrs.	− 3.0	95	3 Hrs.	− 8.1	25
4 Hrs.	− 3.5	95	4 Hrs.	−12.0	25
6 Hrs.	− 7.5	85	6 Hrs.	−16.0	0
(b) exposed in air					
½ Hr.	−11.0	75	½ Hr.	−15.0	40
1 Hr.	−11.0	20	1 Hr.	−15.0	0
1½ Hrs.	−11.2	5	1½ Hrs.	−15.0	0
2 Hrs.	−11.5	5	2 Hrs.	−15.3	0
2½ Hrs.	−11.8	0	2½ Hrs.	−16.2	0
3 Hrs.	−12.4	0	3 Hrs.	−16.0	0

The resistance of eggs and embryos to low temperature was tested under more natural conditions when, on December 5, 1955, 26 egg cases, 11 of which contained ova to segmented stages and 15 contained veligers and shelled veligers, were dredged from New Haven Harbor. The bottom temperature was 7.1° C. and the veligers were observed revolving within their capsule. These cases were placed in tidal tanks in water of 7.5° C. on December 6, 1955, and the veligers were still motile on February 16, 1956, 63 days later. The average water temperature during this period was 2.3° C. with a range from − 0.1° to 7.5° C. By March 22, however, the ova and segmented stages within the cases were disintegrated and the veligers were dead.

To supplement these observations 120 egg cases collected from the laboratory aquaria were conditioned gradually to the outdoor water temperature of 1.3° C. and placed in the Harbor on January 7, 1956. These egg cases were kept until July 20, 1956, but showed no evidence of development.

Discussion

The incubation times found in our experiments are in general agreement with the data of other authors. Thus, Haskin's (1935) report of 18 to 25 days as the time for the first young conchs to hatch at 23.3° to 29.1° C. agrees with our 22-day period at 25.0° C. Cole's (1942) findings of 27 to 32 days at 22.6° C. and 44 to 50 days at 18.3° C. for drills are again in agreement with our observations. If we interpolate our data, we obtain 30 to 38 days at 22.5° C. and 43 to 56 days at 17.5° C.

Compared to our results, Cole (1942) found that the surprisingly short period of only five days was required for all the young conchs to be released at 22.6° C. and only six days at 18.3° C. Cole does not give the number of egg cases used in this experiment and the short period he records may be the result of having very few egg cases, since we found considerable variation between egg cases at any one temperature.

Pope (Carriker, 1955) found that the period for protoconch release from an egg case or group of egg cases, which we assume were deposited at the same time and kept under identical conditions, extended from four to 38 days. He attributes this to an uneven development of the embryos, but we found the embryonic development within a single egg case to be generally uniform, except for malformed embryos and undeveloped ova. By the time of release, some of these are partly eaten by the normally developing drills or remain undeveloped to such an extent that they never emerge (Fig. 4). Considerable variation in the period of incubation, however, does occur between the egg cases deposited within a 16-hour period.

We observed that the greatest variation in hatching time between egg cases occurs after the protoconch stage, which may not be caused entirely by an uneven development of the embryos. Some protoconchs remain in their cases longer than others, which may be due to a variation in some mechanism releasing the egg case operculum. Variations in hatching may also be caused by the physical obstruction of the operculum opening which a protoconch may find too small. On one occasion we saw a young drill, emerging from a case, become entrapped in the operculum opening and block the passage for the remaining protoconchs for eight days.

Our experiments showed that although egg cases can withstand sub-freezing water temperatures for short periods, they cannot survive prolonged periods of chilling. At first it appears to be more drastic to subject the egg cases to sub-freezing temperatures rather than to temperatures above freezing but if, for example, the permeability of the egg cases is affected, the osmotic malfunction progresses at a faster rate at chilling temperatures than at sub-freezing temperatures (Luyet and Gehenio, 1940). Chilling temperatures, as these authors point out, become lethal to protoplasm only after long periods. This may explain the survival of the egg cases which we kept in the tidal tanks for a period of 63 days and also the presence of egg cases observed by other workers during the winter months (Carriker, 1955).

The author wishes to thank Dr. V. L. Loosanoff for his suggestions and constructive criticism throughout the experimental work and both Dr. V. L. Loosanoff and Mr. H. C. Davis for the critical reading of this paper. I also wish to thank Mr. C. A. Nomejko for preparing the photomicrographs and tables.

SUMMARY

1. The rate of ova development increases directly with the increase in temperature from 15.0° to 25.0° C. No increase in the rate of ova development was observed above 25.0° C.

2. Optimum temperature for ova development of *U. cinerea* of Long Island Sound appears to be 20.0° C., or between 20.0° and 25.0° C.

3. Egg cases of *U. cinerea* kept in sea water can withstand sub-freezing temperatures for longer periods than egg cases exposed to sub-freezing air temperatures.

4. In sub-freezing temperatures, the percentage mortality increases with the period of exposure and with a decrease in temperature.

5. Egg cases kept at 10.0° C. for as long as 84 days showed partial development and were capable of producing normal protoconchs when returned to 20.0° C.; whereas, egg cases kept at 7.5° C. for 54 days were not viable.

6. Our experiments and observations suggest that egg cases remaining through the winter in Long Island Sound will not contain viable ova in the spring.

LITERATURE CITED

Carriker, M. R., 1955. Critical review of biology and control of oyster drills, *Urosalpinx* and *Eupleura*. *Sp. Sci. Rept., Fish.* 148.

Cole, H. A., 1942. The American whelk tingle, *Urosalpinx cinerea* (Say), on British oyster beds. *J. Mar. Biol. Assoc.*, **25**: 477–508.

Federighi, H., 1931. Studies on the oyster drill (*Urosalpinx cinerea*, Say). *Bull. U. S. Bur. Fish.*, **47**: 85–115.

Haskin, H. H., 1935. Investigations on the boring and reproduction activities of oyster drills, *Urosalpinx cinerea* (Say) and *Eupleura* sp. *Unpub. Rept. U. S. Bur. Fish.*

Loosanoff, V. L., 1949. Method for supplying a laboratory with warm sea water in winter. *Science*, **110**: 192–193.

Luyet, B. J., and P. H. Gehenio, 1940. Life and death at low temperatures. Biodynamica, Normandy, Missouri.

THE UPTAKE OF RADIOACTIVE CALCIUM BY SEA URCHIN EGGS. I. ENTRANCE OF CA^{45} INTO UNFERTILIZED EGG CYTOPLASM[1]

SIDNEY C. HSIAO AND HOWARD BOROUGHS

Department of Zoology, University of Hawaii and Hawaii Marine Laboratory, Honolulu, T. H.

In studying the physiology of calcium accumulation of sea urchin eggs and its ultimate utilization in the formation of the calcareous skeleton, the use of radioactive calcium as a tracer offers many advantages. But a search of the literature on the uptake of radiocalcium by sea urchin eggs revealed only one report which was a study using an indirect method (Rudenberg, 1953). In his study of the role of the jelly coat in the uptake of calcium by the eggs of *Arbacia punctulata* Rudenberg observed the radioactivity present in his samples by a Geiger tube placed above the incubation medium. He concluded that Ca^{45} was accumulated only by eggs with jelly coats and for up to six hours after fertilization. About six hours after fertilization a loss of calcium from the eggs was noted. But eggs without jelly coats showed no uptake and no loss of calcium. In the present study, experiments have been designed to give more direct answers to the following series of questions: (1) Will sea urchin eggs, with and without jelly coats, take up radiocalcium from the medium? (2) If either or both types of eggs take up radiocalcium from the medium, are the Ca^{45} ions simply adsorbed on the surface or can they be demonstrated in the egg cytoplasm? (3) If there is an uptake of radiocalcium, is it due to a net accumulation of calcium or simply to an exchange of Ca^{45} with stable Ca^{40}?

MATERIALS AND METHODS

Mature, unfertilized eggs of *Tripneustes gratilla* (Linnaeus), a large, common species of sea urchin in Hawaiian waters, were used. Freshly collected females were induced to shed their eggs by the KCl injection method (Tyler, 1949). One female could provide 30–50 ml. of eggs, more than enough for a whole series of experiments. The eggs were strained through bolting cloth or several layers of cheesecloth to remove extraneous matter, and washed with filtered sea water with the help of gentle centrifugation before use in the following experiments.

(1) *Comparison of Ca^{45} uptake by intact eggs and by eggs without jelly coats.* To compare the time course of the uptake of radiocalcium by these two types of eggs, a freshly washed batch of eggs from one female was divided into two approximately equal aliquots. One aliquot was treated with HCl to remove the jelly coats by the method of Harvey (1941). A stock Ca^{45} solution with an activity of 5 μc/ml. was made by dilution of Oak Ridge Ca^{45}Cl$_2$ solution having a specific activity of 73.81 mc/g. with an appropriate quantity of filtered normal sea water. One-half ml. of a 1:10 suspension of eggs with jelly coats removed was put into a 50-ml.

---

[1] Contribution no. 100 from Hawaii Marine Laboratory, University of Hawaii.

beaker already containing 4 ml. of filtered sea water. One-half ml. of the stock Ca^{45} solution was then added, giving a final volume of 5 ml. and a Ca^{45} concentration of 0.5 μc/ml. Twelve beakers were similarly prepared. The content of the first to the twelfth beaker was incubated at room temperature (24–27° C.) with occasional stirring according to the following schedule:

Beaker No.	1	2	3	4	5	6	7	8	9	10	11	12
Incubation time in minutes	0	1	2	4	8	16	32	64	128	256	512	1024

At the end of the specified time of incubation, the content of each beaker was centrifuged to separate the eggs from the incubation medium. As it took two minutes to effect this separation, the actual time of contact between the eggs and Ca^{45} varied from 2 to 1026 minutes.

A 0.5-ml. aliquot of the supernatant medium after each time interval was transferred to an aluminum planchette, dried and counted with a thin window (1.4 mg. cm^2) Geiger tube and a commercial scaler. All samples were prepared in triplicate. Dose planchettes were made of the incubation medium without eggs by diluting it ten-fold and triplicate of 0.5-ml. volume used for counting. A convenient aliquot of the incubated eggs from each time interval was transferred from the centrifuge tube, spread on a weighed planchette, dried to constant weight and the activity of the eggs counted in the same way as the medium. After correction for background and for self-absorption the counts were expressed in counts per minute per mg. dry weight. A similar series was run using eggs with jelly coats intact. These two experiments were repeated, but the incubated eggs were washed in normal filtered sea water before being counted for Ca^{45} activity. As a control, eggs from the same female were incubated in normal filtered sea water and prepared and counted in a similar manner.

(2) *Removal of Ca^{45} from eggs.* In order to ascertain whether the activity of eggs incubated in radiocalcium-containing sea water could be removed from the egg surface, radioactive eggs, with and without jelly coats, were washed with (a) sea water, (b) a 0.02 per cent V/V solution in sea water of a commercial surface active wetting agent, "Sterox SK" (supplied by Monsanto Chemical Co.), and (c) a 0.05 per cent W/V solution in sea water of a commercial detergent "Tide," manufactured by Procter and Gamble Co. Samples of eggs without jelly coats and of intact eggs were washed separately by re-suspending the radioactive eggs in 10 ml. of the washing fluid in a centrifuge tube, and stirring them gently for 5 minutes. At the end of 5 minutes a 0.5-ml. aliquot of the well-mixed egg suspension was transferred to a second tube, centrifuged to remove the washing liquid from the eggs which were then spread on a planchette in order to measure egg radioactivity. The 9.5-ml. egg suspension in the original tube was centrifuged at a RCF of 2330 G to pack down the eggs. The sedimented eggs were completely drained by inverting the tube whose inside wall was wiped dry with absorbent tissue. The washing procedure was repeated five times with each sample of eggs.

(3) *Autoradiography of eggs.* Intact eggs and eggs without jelly coats were made radioactive by incubating them in sea water containing Ca^{45}. They were fixed separately with neutral formalin, dehydrated by direct transfer into dioxane (diethylene dioxide) which was changed once. Infiltration was made with two changes of paraffin. Sections of eggs 5 or 10 microns thick were mounted on slides

from some of which the paraffin was removed by xylene, and the remaining material coated with a thin veneer of gelatin and autoradiographs made on Kodak medical (No Screen) x-ray films. Other sections were treated by Townsley's method (personal communication). In this method, which is similar to that worked out by Bélanger and Leblond (1946), the paraffin sections were floated on slides previously coated with gelatin (Kodak photographic inactive gelatin, 0.5% and chrome alum, 0.05% solution) and allowed to attach by drying. After dissolving the paraffin in xylene the sections were run down through grades of alcohol to double glass-distilled water. The slides were next coated with Ilford nuclear research emulsion, type G.5, which was warmed to 38° C., thinned with a little distilled water and applied in a very thin layer with an artist's brush. The coated slides were dried under an electric fan and placed in a slide box made light proof by being wrapped in aluminum foil and black paper. Exposure of the emulsion to the β-radiation from egg sections was completed in a refrigerator and later developed photographically. Examination of the autoradiographs was made with both light and phase contrast microscopes.

(4) *Total calcium determination.* The total calcium content of ordinary and radiocalcium-containing eggs was estimated after the eggs were dried to constant weight. Aliquots of the dried specimens were wet-ashed by the method of Norris and Lawrence (1953). The egg calcium was precipitated with ammonium oxalate according to Holth's (1949) recommendation to exclude magnesium. The precipitated calcium oxalate was quantitatively estimated by flame spectrophotometry with a Beckman model DU spectrophotometer according to the method published in "Application Data for Beckman Instruments" by the manufacturer. A series of eggs having various radioactivities, from zero to 686 cpm per mg. dry weight, was used for analysis.

RESULTS

When exposed to Ca^{45} in the medium eggs with and without jelly coats showed high rates of radiocalcium uptake during the first few minutes. After this initial phase the quantity of Ca^{45} taken up by the eggs increased with the increased time of incubation. Figure 1 represents the results of a typical experiment. Three things are shown by this figure: (1) unfertilized eggs, with and without jelly coats, took up radiocalcium; (2) unfertilized eggs without jelly coats took up more radiocalcium than intact eggs when both were treated similarly, as shown by the heights of curves A and C in contrast to those of curves B and D of this figure. This was true throughout the whole time course at all the different time intervals of incubation used. (3) When the eggs were washed with filtered sea water after incubation and before being placed on the planchettes for counting, both types of eggs retained Ca^{45}, but the activity due to Ca^{45} retained by eggs without jelly coats was much higher than that of intact eggs, as shown by curves C and D. Eggs from the same female but incubated in normal sea water as control showed no radioactivity at all.

Table I shows the results of washing the treated eggs in order to see if the Ca^{45} taken up by the two types of unfertilized eggs can be removed, assuming that the radiocalcium ions are on the surface of the jelly or in the jelly coats in the case of intact eggs, and on the surface of the vitelline membrane in the case of eggs

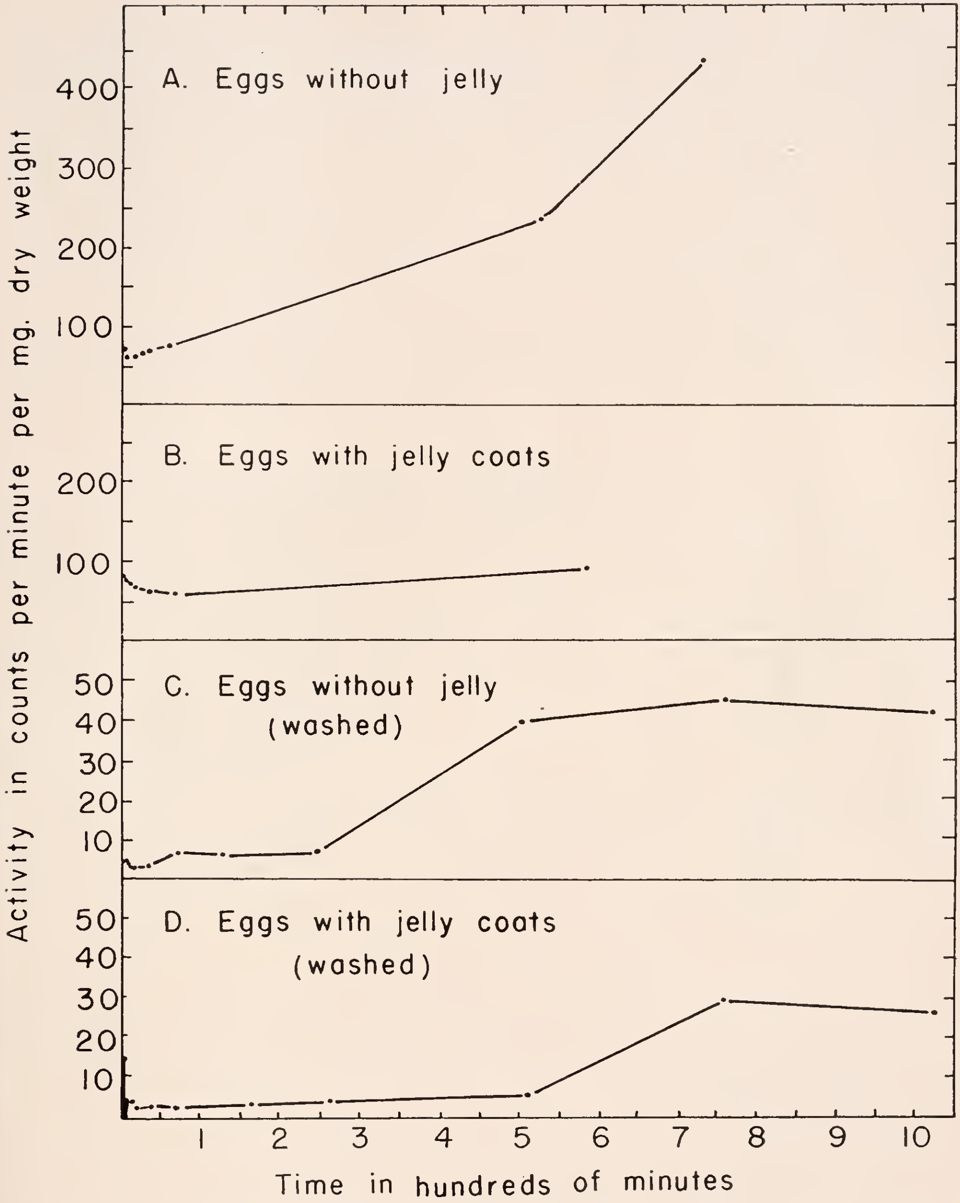


FIGURE 1. Radiocalcium uptake by sea urchin eggs with and without jelly coats for different periods of incubation.

without jelly. It will be seen from this table that eggs without jelly coats had approximately 1.5 times as much activity as intact eggs. Intact eggs washed once with detergent for five minutes lost about 80 per cent of the original radioactivity. On the other hand, eggs without jelly similarly treated lost only about half of the original activity. After the fourth wash the washing fluid showed practically no activity, indicating that a surface active agent could not remove any more Ca^{45} from these eggs. But it will be noticed from Figure 2 that in washing with the wetting agent "Sterox SK," eggs without jelly coats consistently retained more radioactivity after each washing than did eggs with jelly coats. At the end of the fifth washing eggs without jelly coats retained 11 times as much activity as did similarly treated intact eggs, while the washing fluid showed no activity in either case. In washing with a solution of "Tide" (see Table I) about six times as much activity was retained by jelly-free eggs as by eggs with jelly coats. It was noticed during these washing experiments that the first washing of intact eggs removed all the jelly coats.

That the activity remaining in the detergent washed eggs was due to Ca^{45} in the cytoplasm and not removable by surface active agents is shown by autoradiographs of sectioned eggs. In Figure 3 are shown photomicrographs of some of the autoradiographs. Figure 3A is a photomicrograph of an autoradiograph made by three whole unfertilized eggs on an Ilford emulsion. The dark regions around these eggs show the presence of Ca^{45} in the jelly coats. Figure 3B shows an autoradiograph made on x-ray film by an intact egg incubated in Ca^{45}-containing sea water. In this figure the outline of the jelly coat and the egg can be seen. Figure 3C is a lower power photomicrograph made with a light microscope. In the center of this photomicrograph is a section of an untreated egg included among treated eggs whose sections show the presence of Ca^{45} inside the cytoplasm. Figures 3D–3F are photomicrographs made with a phase contrast microscope. In Figure 3D the egg membrane and cytoplasm are seen to contain radiocalcium. Figure 3E is a higher power photomicrograph of the autoradiograph of another group of eggs, while Figure 3F is a detailed view of a single section of a radioactive egg.

TABLE I

The loss of radiocalcium from eggs washed with detergents showing that eggs without jelly coats retained more Ca^{45} than did intact eggs

Material	Washing fluid	Washing No.	Egg activity, cpm	Material	Washing fluid	Washing No.	Egg activity, cpm
Eggs with jelly coats intact	"Wetting agent" 6 drops, sea water 10 ml.	0	499	Eggs without jelly	"Wetting agent" 6 drops, sea water 10 ml.	0	736
		1st	103			1st	321
		2nd	34			2nd	137
		3rd	34			3rd	83
		4th	33			4th	80
		5th	5			5th	56
Eggs with jelly coats intact	"Tide" 1% w/v solution 0.5 ml., sea water 9.5 ml.	0	499	Eggs without jelly	"Tide" 1% w/v solution 0.5 ml., sea water 9.5 ml.	0	736
		1st	121			1st	306
		2nd	31			2nd	199
		3rd	173(?)			3rd	81
		4th	46			4th	82
		5th	9			5th	52

The results of chemical estimation of total calcium content of eggs having taken up various quantities of radiocalcium are shown in Figure 4. This figure shows that of the 15 groups of radioactive eggs analyzed, no group showed significantly different content of total calcium from the average value of all the groups, although the radioactivity of the highest group was about 340 times that of the lowest. It also shows that increase in Ca^{45} taken into the eggs was not followed by increase in total calcium. Total calcium fluctuated independently of the Ca^{45} content of the eggs about a mean value of 2.7 μg per mg. dry weight. The calcium content of ordinary, *i.e.*, unincubated, eggs was not lower than that of radioactive eggs. This indicates that there is no calcium accumulation, but an exchange of ordinary Ca^{40} for radioactive Ca^{45} leaving the total calcium content more or less unaltered.

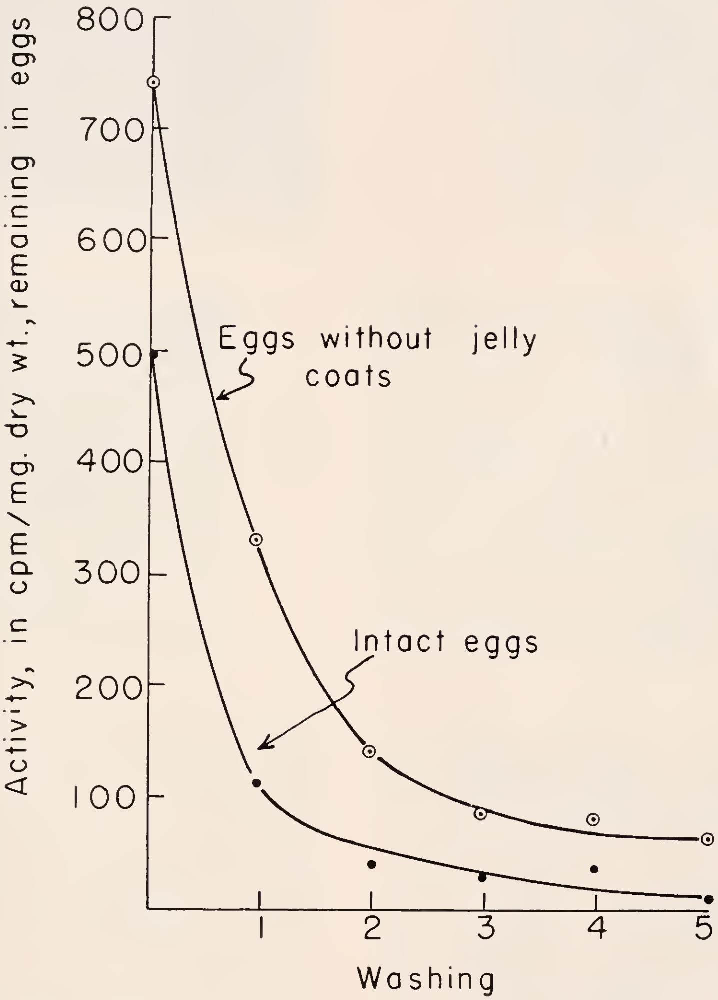


FIGURE 2. Decrease in radiocalcium in sea urchin eggs after washing with detergent solution (Sterox SK).

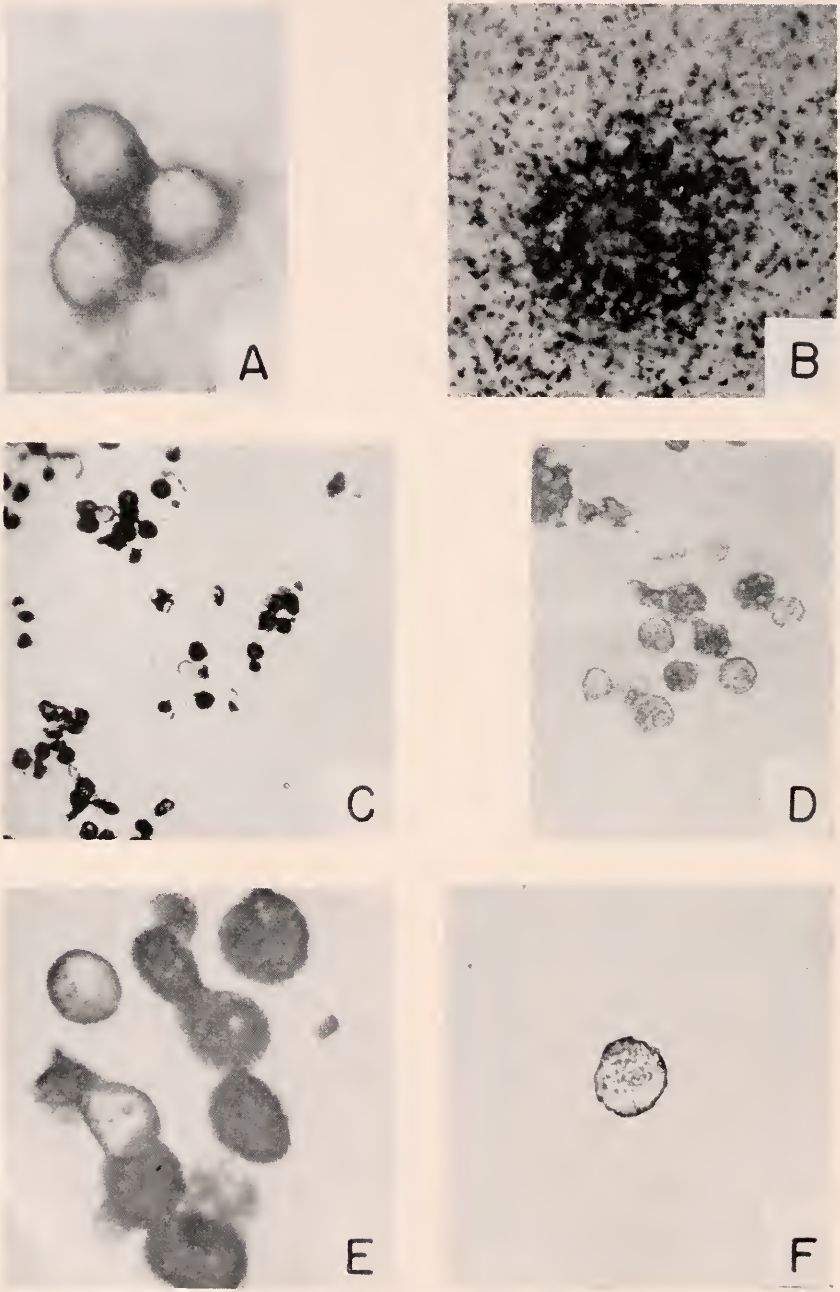


FIGURE 3. Photomicrographs showing Ca^{45} inside egg jelly and in sections of egg cytoplasm. 3A, autoradiograph of three intact eggs showing Ca^{45} among the jelly coats, magnification 100 ×. 3B, autoradiograph on Kodak x-ray film of one intact egg, magnification 150 ×. 3C, autoradiograph of 10 μ thick sections of eggs, magnification 37 ×. 3D, phase contrast photomicrograph of similar egg sections, magnification 70 ×. 3E similar to 3D, magnification 140 ×. 3F, detailed picture of a single section of egg, magnification 140 ×.

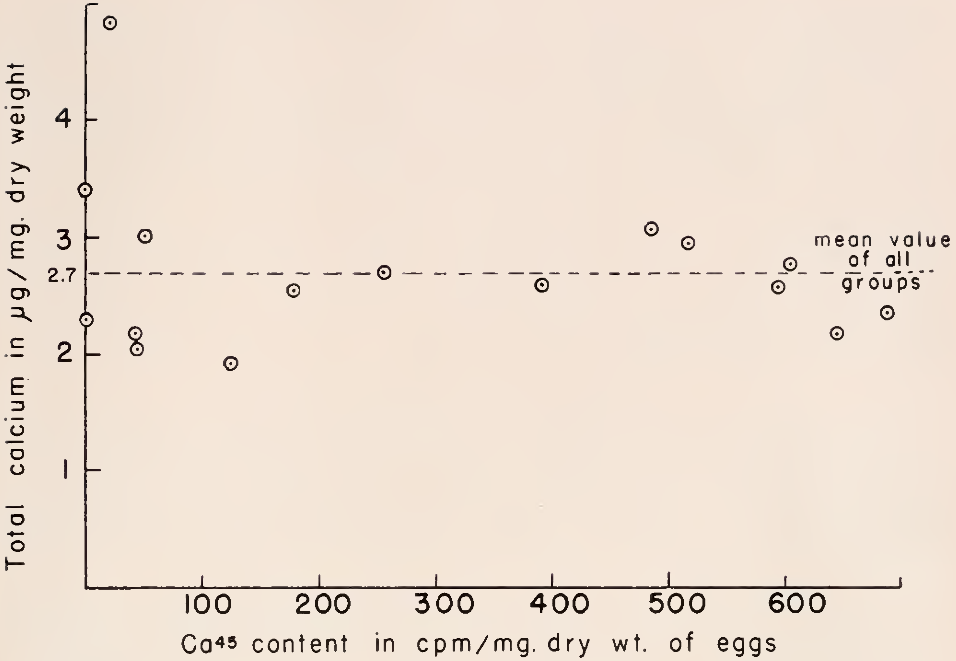


FIGURE 4. Total calcium content of ordinary and radioactive eggs showing no increase in total calcium with increase of Ca^{45} uptake.

DISCUSSION

The consistently greater amount of Ca^{45} taken up by eggs without jelly coats than that by intact eggs indicates that not only is jelly coat unnecessary for the uptake of radiocalcium, but it acts as a hindrance to the entrance of these ions into the egg cytoplasm. As the total diameter of intact eggs, inclusive of jelly coat, measures 126–130 microns, and that of the vitellus about 82 microns, an ion on the surface of the jelly coat would theoretically have to travel 63–65 microns to reach the geometrical center of the egg, while in the case of eggs without jelly, an ion on the surface of the vitelline membrane would have to travel only a distance of 41 microns to reach the same point. It is hence not surprising that for an equal period of incubation in sea water containing radiocalcium, eggs without jelly could take up more Ca^{45} than intact eggs. In other words, in eggs without jelly coats the Ca^{40} of the egg cytoplasm could be exchanged directly with the Ca^{45} in the incubation medium or on the vitelline surface. In intact eggs, the first place of Ca^{45} entry would be the jelly where the entering Ca^{45} could either exchange with the Ca^{40} of the jelly coats or continue to move into the vitellus.

When eggs with jelly coats were washed with detergent, the first washing removed most, if not all, of the jelly. If these eggs were first made radioactive, approximately 80 per cent of the radioactivity came off with the jelly. This observation suggests a possible explanation of the loss of calcium by fertilized *Arbacia* eggs after 6 hours incubation reported by Rudenberg (1953). As the intact radioactive eggs were shaken for 6 or more hours, a good deal of the jelly coats would be detached which, when brought to the surface by stirring, would account for the reported increase in radioactivity in the surface layer of the incubation medium.

Although the autoradiographs of sections of the radioactive eggs do not give the exact location of the Ca^{45} ions in the cytoplasm, because the denaturation of the egg protein by formaldehyde fixation and subsequent histological procedures applied to the eggs and their sections may have altered their positions, the fact that these histological treatments and photographic developments used in connection with Ilford emulsion film coatings did not remove them indicates that the Ca^{45} must be firmly attached to the organic compounds of the eggs. An approximate idea of the site of the uptaken Ca^{45} is provided by the autoradiographs. Further investigations are in progress to determine which fraction or fractions of the egg substance combined with the radiocalcium.

Summary and Conclusions

1. The entrance of radiocalcium into unfertilized eggs of *Tripneustes gratilla* (Linnaeus) has been investigated in this study. By direct measurements of the radioactivity of intact eggs and of eggs without jelly coats it was found that although both types of eggs took up Ca^{45}, eggs without jelly coats took up much more than intact eggs.

2. By washing with detergent both types of eggs made radioactive by incubation in radiocalcium-containing sea water, it was found that jelly-free eggs retained 11 times as much Ca^{45} as intact eggs when washed with a commercial wetting agent "Sterox SK," and 6 times as much when washed with a dilute solution of "Tide."

3. It is concluded that jelly is not essential to the uptake of radiocalcium by sea urchin eggs and the presence of jelly coats reduces the amount of penetration into the egg cytoplasm.

4. Autoradiographs of whole eggs showed the presence of Ca^{45} in the jelly coats if intact eggs were incubated in radiocalcium-containing sea water. Autoradiographs of sections of both types of eggs showed that Ca^{45} was inside the egg cytoplasm.

5. No significant difference in total calcium was found by chemical analysis of groups of eggs incubated for various periods of time in Ca^{45}-containing sea water, *i.e.*, made to take up varying amounts of radiocalcium. None of the groups showed significantly different contents of total calcium from that of ordinary unfertilized eggs. It is concluded that Ca^{45} enters the egg cytoplasm by exchange with Ca^{40} of the eggs.

LITERATURE CITED

Bélanger, L. F., and C. P. Leblond, 1946. A method for locating radioactive elements in tissues with a photographic emulsion. *Endocrinology*, **39**: 8–13.

Harvey, E. B., 1941. Vital staining of centrifuged *Arbacia punctulata* egg. *Biol. Bull.*, **81**: 114–118.

Holth, T., 1949. Separation of calcium from magnesium by oxalate method. A critical study. *Anal. Chem.*, **21**: 1221–1226.

Norris, W. P., and B. J. Lawrence, 1953. Determination of calcium in biological material. *Anal. Chem.*, **25**: 956–960.

Rudenberg, F. H., 1953. The role of the jelly coat in the uptake of calcium by eggs of *Arbacia punctulata* before and after fertilization. *Exp. Cell Res.*, **4**: 116–126.

Tyler, A., 1949. A simple non-injurious method for inducing repeated spawning of sea urchins and sand dollars. *Coll. Net*, **19**: 19–20.

A FUNGUS PARASITE IN OVA OF THE BARNACLE CHTHAMALUS FRAGILIS DENTICULATA

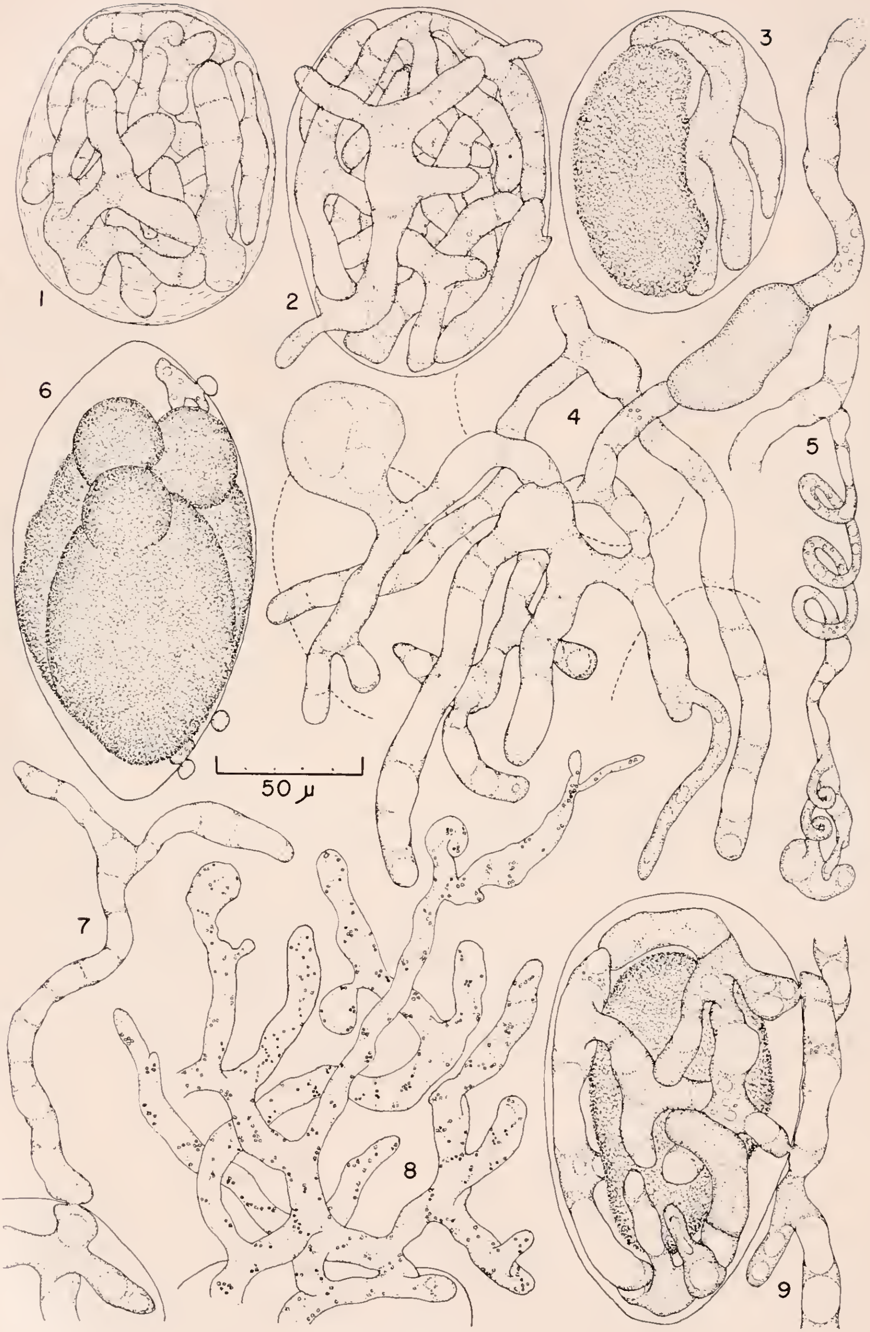
T. W. JOHNSON, JR.

Department of Botany, Duke University, Durham, North Carolina

Species of fungi in marine animals are apparently not numerous, although some are very destructive parasites. Outstanding among the latter are *Ichthyosporidium hoferi* (Plehn and Mulsow, 1911; Sproston, 1944) in herring, salmon, and flounder, and *Dermocystidium marinum* (Mackin *et al.*, 1950; Ray and Chandler, 1955) in oysters. Among other reports of marine zoophagous fungi worthy of mention are: *Cycloptericola marina* in *Cyclopterus lumpus* (Apstein, 1910); *Leptolegnia marina* in the pea crab, *Pinnotheres pisum* (Atkins, 1929, 1954a); a saprolegniaceous and a pythiaceous fungus in the calcareous shells of molluscs (Bornet and Flahault, 1889); *Spongiophaga* sp. in sponges (Galtsoff, 1940); three species of *Nephromyces* in the ductless kidneys of various ascidians (Giard, 1888); a pink yeast (*Torula*) in oysters (Hunter, 1920); boring fungi in various shell-forming animals (Kölliker, 1860); two species of *Thalassomyces* in the decapod *Pasiphaea* (Niezabitowski, 1913); a marine *Laboulbenia* on *Aepus robini* (Picard, 1908); an Ascomycete, *Didymella conchae* (Bonar, 1936) in mollusc shells (a marine lichen according to Santesson, 1939); *Sirolpidium zoophthorum* in lamellibranch larvae (Vishniac, 1955), and unnamed marine eccrinids in *Panopeus herbstii* and *Emerita talpoida* (Wolf and Wolf, 1947). A very few species of fungi occur in the ova of marine invertebrates: *Lagenidium callinectes* (Couch, 1942) in the blue crab, *Callinectes sapidus; Plectospira dubia* and *Pythium thalassium* (Atkins, 1954b, 1955) in the pea crab, and an unnamed fungus suggestive of *Sirolpidium zoophthorum* and *Plectospira dubia* in the oyster drill, *Urosalpinx cinerea* (Ganaros, 1957). Two fungi have been described from barnacles. The Ascomycete *Pharcidia marina* (Santesson, 1939, places this organism in the lichen genus *Arthopyrenia*) occurs on the shells of *Balanus balanoides* (Bommer, 1891); a second Ascomycete, *Didymella balani* (also renamed as a lichen) develops on the test of *Chthamalus stellatus* (Hariot, 1887). A new species of Phycomycete, *Lagenidium chthamalophilum*, parasitic in the ova of *Chthamalus fragilis* var. *denticulata*, is described in this paper.

Lagenidium chthamalophilum sp. nov.

Hyphae crassae, contortae vel irregulares, ramosae; intra- et extramatricales, vacuolis et guttulis multis, pallide flavae usque ad hyalinas; plerumque 10–18 μ in diam. Sporangium ex septatione hyphae formatum, tubulo singulo apice dilato in vesicam sphaericam. Sporae reniformes, a latere biflagellatae, in vesica formantur et vesica deliquescente emittuntur. Oögonia rara; lateralia vel terminalia vel intercalaria sed semper in hyphis intramatricalibus; globosa vel subglobosa vel elongato-irregularia; 19–47 μ diam. Oösporae singulae, vel raro binae; sphaericae


1
2
3
4
5
6
50 µ
7
8
9

FIGURES 1–9.

(21–25 μ diam.) usque ad ellipsoidales (18–27 × 16–23 μ), guttulas centricas vel eccentricas includunt. Germinatio oösporarum adhuc incognita. Antheridia adhuc non observata.

Hyphae stout, contorted or irregular, branched; filling the ova and emergent from them; generally reticulately vacuolate, with numerous minute cytoplasmic oil bodies, infrequently with very diffuse cytoplasm containing oil bodies; pallid golden-yellow to hyaline; occasionally constricted at points of penetration through the egg membrane; variable in diameter, generally 10–18 μ; occasionally producing globose, lateral swellings up to 39 μ in diameter. Sporangia formed by segmentation of intramatrical hyphae, very rarely produced on extramatrical hyphae; variable in length, diameter coincident with that of hypha; each producing one stout emergent discharge tube expanded apically into a spherical vesicle. Planonts reniform, laterally biflagellate, 8.5–10.2 × 6.8–8.5 μ; cleaved from sporangial protoplasm within the vesicle; aplerotic; discharged upon rapid deliquescence of vesicle. Oögonia rare; lateral, terminal, or intercalary on intramatrical hyphae; globose, subglobose, or slightly elongate-irregular; 19–47 μ in diameter. Oöspores 1, rarely 2; spherical, blunt-conic, or nearly ellipsoid; containing a single centric or eccentric mass of small oil globules; 18–27 × 16–23 μ, spherical ones 21–25 μ in diameter; germination unknown. Antheridia not observed.

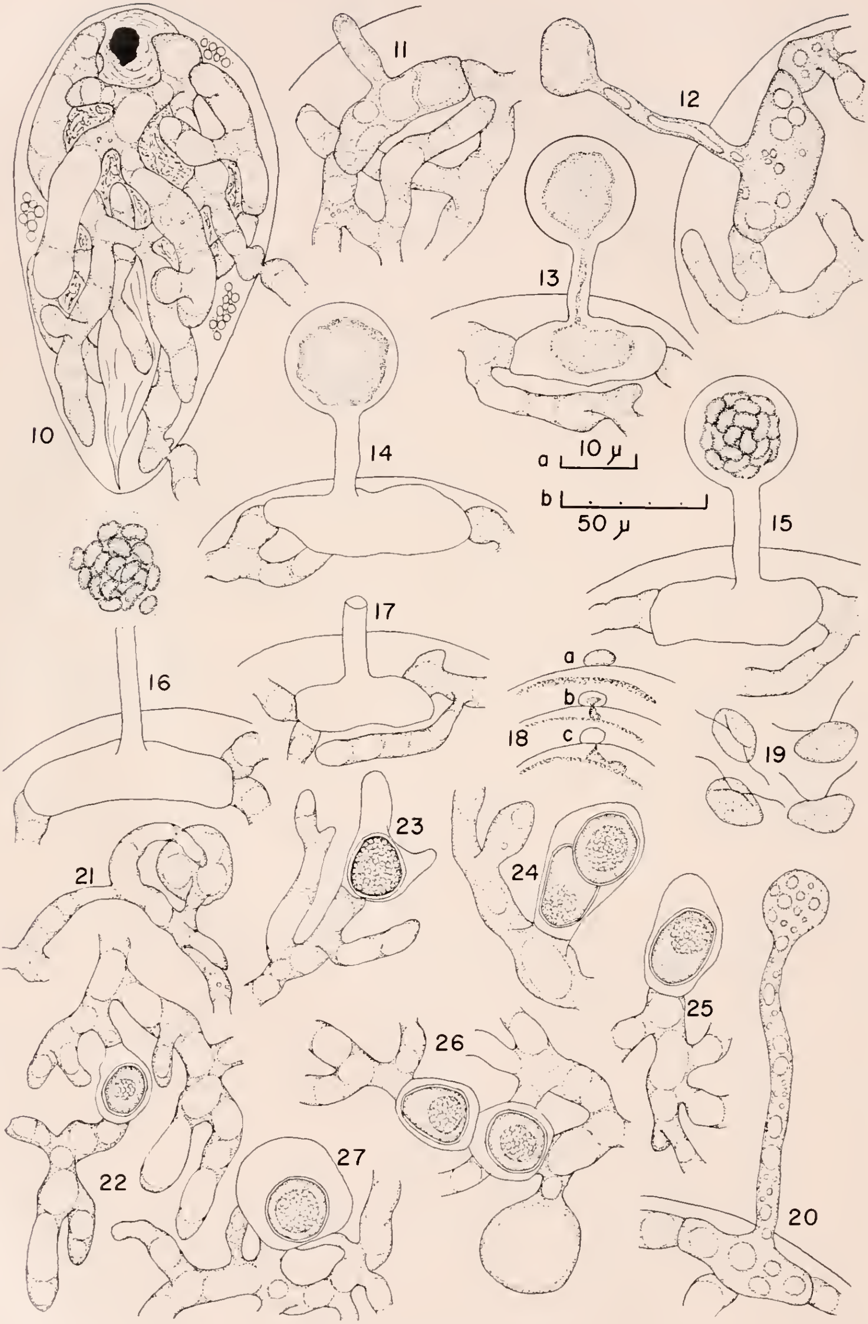
Parasitic in ova of *Chthamalus fragilis denticulata,* Beaufort Inlet, North Carolina, June 17, 1957 (TYPE), leg. J. D. Costlow.

Chthamalus fragilis denticulata, common in the Beaufort, North Carolina region, is a small, pallid-brown to grayish-white, sessile barnacle attaching to pilings, rocks, sea walls, to other barnacles, and to the stems and leaves of *Spartina alterniflora.* The animal occurs only on the uppermost portions of pilings, for example, near the high water line. These barnacles, among the last to be covered by water at incoming tide, are submerged for the short slack high water period, and are the first to be exposed on ebb tide. *Chthamalus fragilis denticulata* occurs with, in fact often attaches to, a second equally abundant barnacle, *Balanus amphitrite.* The lamellae (egg cases) of *C. fragilis denticulata* are usually paired and lie free within the mantle. The larval planktonic stage, the nauplius, develops and is liberated within the parent barnacle. Uninfected eggs (in mass) change color as the embryo develops: bright orange-yellow, pallid yellow, pale cream. Infected lamellae, however, are often pallid gray or grayish-green.

Lagenidium chthamalophilum may develop in the ova of *Chthamalus fragilis denticulata* at any time between late gastrulation and emergence of the nauplii. Released nauplii are apparently not infected, nor are there any somatic tissues of the parent animal invaded by the fungus. Ova showing three or more appendage buds are generally most often infected (Fig. 6). Early stage embryos (one or more appendage buds) in entire egg masses may be destroyed, leaving only a cluster of egg membranes filled with fungus mycelium (Figs. 1, 2). On the other hand, if lamellae with more mature or differentiated embryos within the egg mass become infected, some embryos escape invasion by the fungus and develop into

---

FIGURES 1–9. *Lagenidium chthamalophilum.* 1, 2, hyphae within ova membrane; 3, early infection stage showing branching of hypha; 4, emergent hyphae with a sporangial and oögonial initial; 5, coiled extramatrical hypha; 6, infection by two spores of an embryo with three appendage buds; 7, vacuolate extramatrical hypha; 8, guttulate extramatrical hypha; 9, intra- and extramatrical hyphae showing constrictions and two sporangial initials.


10
11
12
13
14
a 10 μ
b 50 μ
15
16
17
a
b
c
18
19
21
23
24
25
20
26
22
27

Figures 10–27.

the planktonic stage. Infection visible in one or two peripheral ova in a lamella spreads rapidly through the entire cluster so that within two days (continual submersion in raw sea water) all embryos are invaded.

Inoculation is brought about by laterally biflagellate planonts. After a 10–15 minute period of active swimming (in 50 ml. of raw, aerated sea water, at 25° C.) the spores settle on an ovum (Fig. 18a) without rounding up. Within three minutes after attaching to the egg membrane, the spore protoplasm penetrates the membrane (Fig. 18b), enlarges rapidly into a foot-like hyphal rudiment (Fig. 18c), and grows along the embryo. Within 25 minutes after inoculation, infection has been established and the young hypha is developed (Fig. 6). Whether hyphae actually penetrate the embryo is not known; the dense, opaque embryonic host cells prevent direct observation, and a suitable technique for fixing and sectioning infected eggs has not been developed.

The vegetative hyphae of *Lagenidium chthamalophilum* are very characteristic. Early in the incubation period, the hyphae become highly vacuolate, and generally maintain a reticulate vacuolation throughout development. Hyphae are characteristically "foamy" in appearance, suggestive of those of *Monoblepharis*. Emergent hyphae, similarly, are usually extremely vacuolate (Figs. 4, 7), but occasionally have very diffuse, strand-like cytoplasm (Fig. 8). In either case, the many minute refractive oil bodies in the cytoplasm give it a readily discernible golden-yellow cast. Emergent hyphae (other than the sporangial discharge tubes and vesicles) are not often observed in the lamellae immediately after dissection from the animal. Such hyphae form in abundance, and sporangial discharge occurs frequently, however, when infected lamellae are placed in sterile sea water and incubated for 12–18 hours at room temperature. Hyphae emerging from an ovum penetrate the membrane either with or without constriction (Figs. 2, 10). The extramatrical hyphae are generally stout and somewhat contorted, freely branched, and of a diameter coincident with that of the intramatrical hyphae. Occasionally, the emergent hyphae are very slender and coiled (Fig. 5), as in *Pythium thalassium* (Atkins, 1955). Neither the intra- nor extramatrical hyphae are septate except where reproductive cells are delimited.

Sporangia are formed by segmentation of the intramatrical hyphae almost exclusively. During formation of the delimiting septa, that portion of the hypha destined to become a sporangium accumulates protoplasm, and often has a few large vacuoles (Fig. 12). These vacuoles disappear prior to movement of the protoplasm into the apical vesicle. A stout discharge or exit tube (Fig. 11) develops from the cylindrical sporangium, penetrates the egg membrane (without constriction) and elongates. The apex of the exit tube enlarges to form, at first, a subglobose or slightly irregular swelling (Figs. 12, 20) containing very diffuse, vacuolated cytoplasm (Fig. 12). The bulbous discharge tube apex subsequently becomes perfectly spherical; it is completely formed before the sporangial content flows into the tube. The vesicle wall is cellulose as is the basal sporangium and vegetative hypha wall.

FIGURES 10–27. *Lagenidium chthamalophilum.* 10, infection in a pre-emergence embryo with eye spot; 11–17, stages in formation of vesicle and spores, and spore discharge (see text); 18, germination and penetration of spore protoplast; 19, planonts; 20, sporangial discharge tube and immature vesicle; 21, immature oögonium; 22–27, mature oögonia; Fig. 19, scale a, others, scale b.

Sporogenesis begins with movement of the sporangial protoplasm through the discharge tube and into the vesicle. Occasionally, the protoplasmic stream separates partially, leaving one or more fusiform masses connected to the main complement by a slender strand (Fig. 13). The sporangial content and the cytoplasm of the tube aggregate into a slightly irregular mass centrally located in the vesicle (Fig. 14). Spores are cleaved out in the protoplasmic mass, appearing first as polygonal units, then as definite spherical or reniform cells (Figs. 15, 16). In no instances observed did the spores fill the vesicle.

Spore discharge is initiated with a slow "shimmering" motion of the vesicular spore mass. The movement then becomes undulating and increases in rapidity until the spores are moving rapidly over and around one another in the center of the vesicle. For one or two minutes the spores are moving extremely rapidly. If such spores are killed with osmic acid fumes and stained with acid fuchsin or gentian violet, short, stubby flagella are visible on the peripheral spores. The vesicle deliquesces (Fig. 16) within 30 seconds, leaving the rapidly moving spores hanging together momentarily. One or two peripheral spores dart away, and subsequently, within a few seconds the spore mass breaks up to liberate rapidly but evenly swimming spores. The entire process, from migration of the undifferentiated protoplasm to spore discharge, is completed within 20 minutes, in raw, aerated sea water at 25–27° C.

Sexual reproductive cells are rarely produced *in vivo*. Short lateral branches with enlarged apices mature into oögonia containing a single oöspore (Fig. 27), but oögonia may also develop as intercalary (Figs. 22, 26) or terminal (Figs. 23, 25) hyphal segments. Intercalary or terminal oögonia often contain two oöspores. Whether antheridia are produced by *L. chthamalophilum* is not known; hypogynous antheridial cells, certainly, are not in evidence. In a few instances, short hyphae were observed near oögonia and in contact with them (Figs. 21, 27), but no antheridial cells were evident. These hyphal branches may be nonfunctional antheridial branches; if so, they are of monoclinous and androgynous origin (Johnson, 1955, pp. 14, 15). Extramatrical hyphae do not produce sex cells.

Attempts to culture *Lagenidium chthamalophilum* were successful. Spores germinated well on aged sea water agar, and on sea water agar fortified with 0.1% glucose and 0.05% yeast extract. Subsequent growth was very sparse, though extensive, and neither sexual nor asexual cells developed in culture. Very slender, sparingly branched, contorted, vacuolate hyphae are produced on the agar media.

The parasite can, in my opinion, be assigned equally well to *Pythium* (Middleton, 1943) or *Lagenidium* (see Sparrow, 1943) as these genera are presently understood and circumscribed. In both genera, planont maturation occurs, generally, in an evanescent vesicle produced at the apex of a sporangium or sporangial discharge tube. In neither genus, however, is the vesicle pre-formed. This fact alone, were it to be considered significant at the generic level, would exclude the barnacle parasite from both genera. On the other hand, the evidence is stronger in favor of assigning the fungus to *Lagenidium* than to *Pythium*. The lagenidiaceous features of the parasite are: the "foamy," granular cytoplasmic content of the stout, branched hyphae; sporangial delimitation by hyphal segmentation, and oöspore formation by contraction of hyphal segment content. The nonseptate nature of the hyphae, of course, suggests *Pythium* rather than *Lagenidium*, although members of the latter genus having pythiaceous mycelium are known (Sparrow, 1943). The fungus in barnacle ova, while suggestive vegetatively of the *Aphragmium* type of *Pythium,* has a simplified sexual apparatus, that is, no well defined antheridium, and no periplasm in the oögonium. In the final analysis, assignment of the fungus to *Lagenidium* turns, I believe, on simplicity of the sexual structures.

Two marine species of *Lagenidium* are known. *Lagenidium sp.* (Johnson, 1957) produces sporangia formed by hyphal segmentation just as does *L. chthamalophilum,* but the hyphae of the former are not stout and vacuolate, and the vesicle is not pre-formed. These two features also separate *L. chthamalophilum* from *L. callinectes* (Couch, 1942), parasitic in ova of *Callinectes sapidus.* Furthermore, *L. callinectes* has a persistent vesicle, the barnacle parasite does not. *Lagenidium chthamalophilum* differs in several significant respects from all other known species in the genus. The irregular, contorted, stout hyphae (with lateral lobulations) of *L. entophytum* (Pringsheim) Zopf suggests *L. chthamalophilum,* but other features separate the two immediately. *Lagenidium closterii* deWildeman produces an extramatrical sporangial discharge tube, as in *L. chthamalophilum;* the hyphae of the former are more delicate and the discharge tube is bulbous at the base. The pythiaceous hyphae of *L. marchalianum* deWildeman are very slender and markedly constricted; these differ significantly from the stout, vacuolate hyphae of *L. chthamalophilum.* The only other myceloid member of *Lagenidium* suggestive of the barnacle parasite is *L. giganteum* (Couch, 1935). Couch's species, however, has segmented mycelium, and lacks a pre-formed vesicle.

Vegetatively, *Lagenidium chthamalophilum* resembles *Plectospira dubia* (Atkins, 1954b), particularly in the stout, irregularly branched and swollen hyphae. In other characteristics, notably those of sporogenesis and discharge, these two fungi are obviously dissimilar. *Pythium thalassium,* parasitic in *Pinnotheres pisum* ova (Atkins, 1955), produces very stout hyphae resembling those of *L. chthamalophilum,* but the *Pythium* has two major characteristics distinguishing it from the barnacle parasite: the sporangia of *P. thalassium* are filamentous and proliferate internally, and the hyphae are not highly vacuolate.

The geographical distribution of *Lagenidium chthamalophilum* is not known, inasmuch as only host barnacles in the immediate vicinity of the Duke Marine Laboratory have been examined. Forty-four collections (totalling 1284 individuals) of *Chthamalus fragilis denticulata* were made within a five-mile radius of the Laboratory, including two series of collections on the outer banks of the Inlet region. The number of egg-bearing parent barnacles, and the number of infected lamellae in any one collection varied considerably. A sample series of ten collections, showing infection incidence, is given in Table I (each animal with paired lamellae). Occasionally, barnacles were collected in which only one egg mass was found. Twenty-one per cent of such masses were infected. It should be noted that some infected lamellae may have been overlooked, especially if they had been inoculated shortly before the animals were collected. For example, in eight dissections out of twenty-nine, visually uninfected ova masses showed infection after three days in storage in sterile, filtered sea water. Thirty-four per cent of all examined *C. fragilis denticulata* lamellae (1016 individual cases) were infected. Percentages of infection are based on hosts collected from pilings and mooring stakes. The same species of barnacle occurring on *Spartina alterniflora* showed some infection, but only infrequently. Only 3 of 86 barnacles (with one or two lamellae) from *Spartina* were invaded by the *Lagenidium.*

A replicated experiment was performed to determine whether *Lagenidium chthamalophilum* is actively parasitic in ova of *Chthamalus*, or is an invader of moribund eggs. Egg masses were dissected from the barnacles with sterilized needles, examined immediately with a dissecting microscope, and separated into two lots, infected and uninfected. As each lamella was removed, it was placed in a drop of sterile sea water to eliminate inadvertent inoculation in handling infected and uninfected eggs. The obviously parasitized egg masses were easily detected with the dissecting microscope. Infected lamellae were placed in small Petri plates, covered with sterile sea water, and incubated overnight. This short period of incubation induced sporulation. The uninfected lamellae were kept in pairs, as they were dissected from the parent animal, and incubated for 12–24 hours in drops of sterile sea water on slides in damp chambers. The masses were then examined; if no infection was visible, one lamella of the pair was placed in the dish with the sporulating fungus, the other lamella (from the same animal) in a separate Petri plate of sterile sea water, and utilized as the control. No specific stages of embryo development were selected for the tests. The plates were incubated at room temperature, and examined at 1, 3, and 5 days. Some plates containing a parasitized and nonparasitized egg mass were discarded when the control lamella (one of the uninfected pair) showed signs of the fungus.

TABLE I

Incidence and percentage infection of Chthamalus fragilis denticulata lamellae by Lagenidium chthamalophilum

No. animals in sample	No. animals with ova	No. of lamellae	No. infected lamellae	Percentage infection
41	14	28	8	28.5
34	31	62	42	67.7
15	15	30	28	93.3
13	12	24	16	66.6
43	37	74	54	72.9
22	3	6	6	100.0
11	6	12	2	16.6
41	40	80	62	77.5
65	49	98	84	85.6
23	8	16	2	12.5

No infection was visible in the "uninfected" egg cases by the end of 24 hours. At 72 hours, however, all naturally uninfected lamellae had been invaded by *Lagenidium chthamalophilum*. Two controls showed infection, and three lamellae in the dishes with the fungus had matured into nauplii; these plates were discarded. In sea water, in the laboratory, visible infection develops between 24 and 72 hours; inoculation, presumably, may occur during the first 24-hour period. Under natural conditions, inoculation must occur (since the "inoculum" is a motile spore) during the short periods (twice in approximately 24 hours) that the opercula of the barnacle are open while the animal is submerged at high tides. This period of time varies roughly—during the neap tide periods, at least—between 45 and 90 minutes, diurnally, for those individuals highest on the substratum. It is true that an exposed, closed animal retains sufficient water within the mantle to enable the planonts of the fungus to swim about and presumably cause infection. This seems a less likely time for infection to occur, however, since tests show that heavily fouled, oxygen-depleted sea water has a retarding effect on spore discharge and movement.

The close natural association of *Chthamalus fragilis denticulata* and *Balanus amphitrite* prompted a replicated series of artificial inoculations using infected lamellae from *C. fragilis denticulata* and uninfected masses from *B. amphitrite*. Lamellae were dissected and incubated in the manner described previously. Forty-three attempts at inducing infection in *B. amphitrite* ova were made. No infection in *B. amphitrite* eggs was evident at the end of 21 days, although the infected ova of *C. fragilis denticulata* were completely destroyed at the end of the three-week incubation period. While some lamellae of *B. amphitrite* were actually inoculated with planonts of the *Lagenidium* (visual inspection of individual ova), these spores did not germinate, or, if they germinated, did not penetrate the egg membrane. Many *B. amphitrite* egg masses were examined from animals collected in the same localities as *C. fragilis denticulata*, but none was infected. Dr. J. D. Costlow has never observed infection in the lamellae of *B. amphitrite*, although he has used ova from this species in extensive studies on larval development. These observations, in view of the proximity of *B. amphitrite* and *C. fragilis denticulata* in natural habitats, suggest the hypothesis that the ova of the former are, if not immune, certainly highly resistant to *L. chthamalophilum*.

The importance of *Lagenidium chthamalophilum* in reducing *Chthamalus fragilis denticulata* populations cannot be judged from this preliminary investigation. Certain further studies on the fungus and its host, however, may be of significance in elucidating the effect of the fungus. Significant among these studies are: distribution and severity of infection; conditions favorable to establishment and spread of infection; the period, in the reproductive cycle of the host, at which the animal is most susceptible; any fluctuations (and causes thereof) in percentage of infection, and a search for other suscepts.

The support of the National Science Foundation, through Grant G-2324, is gratefully acknowledged. I am indebted to Dr. J. D. Costlow, Duke University Marine Laboratory, for technical guidance in the zoological aspects, and to several of my colleagues for opinions and criticisms of the mycological portions of the investigation. Mr. Thomas M. Simkins, Jr., Duke University Library, very kindly prepared the Latin diagnosis.

Summary

1. *Lagenidium chthamalophilum* is described as a parasite of the ova of *Chthamalus fragilis denticulata*. The pathogen is characterized by the formation of a vesicle before sporangial protoplasm migration, and by highly vacuolate, stout vegetative hyphae. In these features, *L. chthamalophilum* differs from the usual interpretation of members of the genus. The fungus is compared with other Phycomycetes known to parasitize crustacean ova.

2. Artificial inoculation experiments show *L. chthamalophilum* to be specific for *C. fragilis denticulata*. The associated barnacle, *Balanus amphitrite*, is resistant to the fungus.

LITERATURE CITED

Apstein, C., 1910. *Cyclopterus lumpus,* der Seehase. Seine Fischerei und sein Wageninhalt. *Mitteil. Deutsch. Seefischerei-Vereins,* **26**: 450–465.

Atkins, D., 1929. On a fungus allied to the Saprolegniaceae found in the pea-crab *Pinnotheres. J. Mar. Biol. Assoc.,* **16**: 203–219.

Atkins, D., 1954a. Further notes on a marine member of the Saprolegniaceae, *Leptolegnia marina* n. sp., infecting certain invertebrates. *J. Mar. Biol. Assoc.,* **33**: 613–625.

Atkins, D., 1954b. A marine fungus *Plectospira dubia* n. sp. (Saprolegniaceae), infecting crustacean eggs and small crustacea. *J. Mar. Biol. Assoc.,* **33**: 721–732.

Atkins, D., 1955. *Pythium thalassium* sp. nov. infecting the egg-mass of the pea-crab, *Pinnotheres pisum. Trans. Brit. Myc. Soc.,* **38**: 31–46.

Bommer, C., 1891. Un champignon pyrénomycète se développant sur le test des Balanes. *Bull. des Séances Soc. Belg. Micr.,* **17**: 151–154.

Bonar, L., 1936. An unusual Ascomycete in the shells of marine animals. *Univ. California Publ. Bot.,* **19**: 187–194.

Bornet, E., and C. Flahault, 1889. Sur quelques plantes vivant dans le test calcaire des Mollusques. *Bull. Soc. Bot. France,* **36**: CXLVII–CLXXVII.

Couch, J. N., 1935. A new saprophytic species of *Lagenidium,* with notes on other forms. *Mycologia,* **27**: 376–387.

Couch, J. N., 1942. A new fungus on crab eggs. *J. Elisha Mitchell Sci. Soc.,* **58**: 158–162.

Galtsoff, P. S., 1940. Wasting disease causing mortality of sponges in the West Indies and Gulf of Mexico. *Proc. 8th Amer. Sci. Cong.,* **3**: 411–421.

Ganaros, A. E., 1957. Marine fungus infecting eggs and embryos of *Urosalpinx cinerea. Science,* **125**: 1194.

Giard, A., 1888. Sur les *Nephromyces,* genre nouveau de champignons parasites du rein des Molgulidées. *C. R. Acad. Sci., Paris,* **106**: 1180–1182.

Hariot, P., 1887. Note sur le genre *Mastodia. J. de Bot.,* **1**: 231–234.

Hunter, A. C., 1920. A pink yeast causing spoilage in oysters. *U. S. D. A. Bull. No. 819,* pp. 1–24.

Johnson, T. W., Jr., 1955. The Genus *Achlya:* Morphology and Taxonomy. 180 pp. Univ. Mich Press: Ann Arbor.

Johnson, T. W., Jr., 1957. Marine fungi. III. Phycomycetes. *Mycologia,* **49**: 392–400.

Kölliker, A., 1860. On the frequent occurrence of vegetable parasites in the hard tissues of the lower animals. *Quart. J. Micr. Sci.,* **8**: 171–187.

Mackin, J. G., H. M. Owen and A. Collier, 1950. Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp., in *Crassostrea virginica* (Gmelin). *Science,* **111**: 328–329.

Middleton, J. T., 1943. The taxonomy, host range and geographic distribution of the genus *Pythium. Mem. Torrey Bot. Club,* **20**: 1–171.

Niezabitowski, E. L., 1913. Pasorzyty roślinne morskich raków głębinowych z rodzaju *Pasiphaea.* (Die pflanzlichen Parasiten der Tiefsee-Decapoden-Gattung *Pasiphaea*). *Kosmos,* **38**: 1563–1572.

Picard, F., 1908. Sur une Laboulbéniacée marine (*Laboulbenia marina* n. sp.) parasite d'*Aepus Robini* Laboulbène. *C. R. Seances et Mem. Soc. Biol., Paris,* **65**: 484–486.

Plehn, M., and M. Mulsow, 1911. Der Erreger der "Taumelkrankheit" der Salmoniden. *Centrabl. Bakt. Parasitenk.,* **59**: 63–68.

Ray, S. M., and A. C. Chandler, 1955. *Dermocystidium marinum,* a parasite of oysters. *Exp. Parasitol.,* **4**: 172–200.

Santesson, R., 1939. Amphibious Pyrenolichens I. *Arkiv för Bot.,* **29A**: 1–67.

Sparrow, F. K., Jr., 1943. Aquatic Phycomycetes exclusive of the Saprolegniaceae and *Pythium.* 785 pp. Univ. Michigan Press: Ann Arbor.

Sproston, Nora G., 1944. *Ichthyosporidium hoferi* (Plehn & Mulsow, 1911), an internal fungoid parasite of the mackerel. *J. Mar. Biol. Assoc.,* **26**: 72–98.

Vishniac, Helen S., 1955. The morphology and nutrition of a new species of *Sirolpidium. Mycologia,* **47**: 633–645.

Wolf, F. A., and F. T. Wolf, 1947. The Fungi. Vol. II, 538 pp. Wiley: New York.

THE EFFECT OF X-RAYS, IRRADIATED SEA WATER, AND OXIDIZING AGENTS ON THE RATE OF ATTACHMENT OF BUGULA LARVAE[1]

WILLIAM F. LYNCH

St. Ambrose College, Davenport, Iowa, and the
Marine Biological Laboratory, Woods Hole, Mass.

Few observations concerning the effects of ionizing radiations have been made on bryozoan tissues. Oka (1954) x-rayed various regions of the fresh-water bryozoan, *Lophopodella carteri,* and noted that the more active embryonic tissues had greater radiosensitivity than other parts. But no observations known to the writer have been reported concerning the effects of x-radiation on the attachment and metamorphosis of bryozoan larvae.

It is well known that some of the biological effects of ionizing radiations have been attributed to the production of H_2O_2 or organic peroxides when aqueous solutions are x-rayed (Evans, 1947; Barron *et al.,* 1949; Kimball and Gaither, 1952). Barron and his co-workers found that both irradiated sea water and hydrogen peroxide inhibited oxygen uptake in sea urchin sperm; but the latter had two opposite effects, increasing respiration at great dilution and inhibiting oxygen uptake in higher concentration. Furthermore, Blum (1941, p. 96) has emphasized the probability that the effects of certain photodynamic dyes may be mediated through the production of H_2O_2. Since some of the basic dyes had been found to be potent inductors of attachment and metamorphosis of *Bugula* larvae when sea water solutions of these dyes were exposed to light (Lynch, 1955a), it was of interest to determine whether x-rays and hydrogen peroxide would have any effect on setting. If ionizing radiations were found to affect the rate of attachment of the larvae, the problem of determining whether the action of these rays was a direct or an indirect one would naturally arise. After x-raying the larvae proved to have a positive effect on the rate of setting, it was decided to employ sea water seeded immediately after being x-rayed with non-irradiated larvae. The results of these experiments led to an investigation of the possible effects of adding H_2O_2 to sea water and finally to observations on the action of two other oxidizing agents, sodium 2,6-dichlorobenzenoneindophenol and 2,3,5-triphenyltetrazolium chloride, on the attachment and metamorphosis of *Bugula* larvae.

MATERIALS AND METHODS

Each experiment on the effects of radiation involved three dishes: the first contained larvae x-rayed in filtered sea water; the second contained sea water that was seeded immediately after being x-rayed with non-irradiated larvae, and the

---

[1] This investigation was aided by a grant from the National Science Foundation, NSF 1728, and by a scholarship given by the Marine Biological Laboratory, Woods Hole, Massachusetts. The writer is grateful to Dr. Albert Kind for supplying some of the oxidizing agents used for these observations.

third had control larvae in their natural medium. For these experiments plastic containers 7 cm. in diameter and 1.6 cm. high were employed. Each dish contained 30 ml. of sea water and was covered with a plastic top; all three containers were kept close together on a table and were wrapped with paper towels until the time of counting. Light was excluded to delay the decomposition of any photolabile by-products, especially peroxides, that might be formed by irradiation.

The x-ray data are as follows: the machine operates on 182 kv. pk. and 25 ma. with an equivalent filtration of 0.2 mm. of copper. During the summer of 1956, when larvae of *B. turrita* were employed, the output of the tubes (position A) was 4724 r per minute and the organisms were irradiated for three minutes and twenty seconds to give a total of 15,733 r. During the previous summer the x-ray dosage for larvae of *B. flabellata* was 18,333 r and the organisms were also irradiated for three minutes and twenty seconds at 5500 r per minute. The tubes were water-cooled and an electric fan was directed upon them. Since the temperature was found to rise only a fraction of a degree, the irradiated material was not cooled by an ice chamber.

Sea water solutions of the three oxidizing agents were made in the following concentrations found to be most effective: 1:14,000 parts by volume of 30% H_2O_2 (7×10^{-4} *M*), 1×10^{-5} *M* 2,3,5-triphenyltetrazolium chloride (TTC) and 0.01 mg. of sodium 2,6-dichlorobenzenoneindophenol (SDBI) per liter (3.4×10^{-8} *M*). The pH was that of natural sea water. Stender dishes 6 cm. in diameter containing 30 ml. of solution were seeded with larvae and covered with glass lids. The controls were placed in the same amount of sea water in similar containers and kept as near as possible to the experimental dishes. In the experiments with H_2O_2 both experimental and control dishes were shielded from light by wrapping them in paper towels. The others were exposed to diffuse daylight coming from a window about three feet from the region where the Stender dishes were placed.

RESULTS

I. X-rays and irradiated sea water

Table I shows that x-raying larvae of *B. flabellata* induced more rapid setting than that which occurred in the controls, the *t* ratio for the difference of the two groups being 5.12 ($P = .005$). For these experiments the number of attached organisms was counted thirty minutes after irradiation with 18,333 r. The three experiments in which larvae were placed in sea water immediately after it had been irradiated suggest that the accelerated rate of attachment is an indirect effect presumably caused by the action of ionizing radiations on sea water.

Table II gives more convincing evidence of an indirect effect of ionizing radiations. For these experiments larvae of *B. turrita* were irradiated with 15,733 r and the number of attached organisms was counted at the end of eight hours. The time of irradiation was the same for both groups of larvae, but it was found that the output of the x-ray tubes had dropped during the course of a year when the machine was calibrated towards the end of the period of experimentation. Although the rate of attachment of the larvae of both *B. flabellata* and *B. turrita* was accelerated either by x-raying the larvae or by seeding them in irradiated sea water, the time at which the effects could be detected differed considerably. Larvae of

B. turrita showed no notable acceleration of the rate of attachment by thirty minutes after either being x-rayed or being placed in irradiated sea water, but by eight hours there were always more attached organisms in the experimental dishes than in the controls. The *t* ratio for counts made at this time indicates a significant difference in the mean number of attached organisms in the experimental and control dishes, being 6.64 ($P = .001$) for irradiated larvae and 4.09 ($P = .005$) for organisms seeded in irradiated sea water. It does not appear that the difference in the rate of setting of *B. flabellata* and *B. turrita* can be ascribed to the lower x-ray dosage of the latter, for 20,000 r did not produce effects appreciably different from those which followed irradiation with 15,733 r. After studying the effects of various agents on both types of larvae, one gains the general impression that it is both more difficult to induce metamorphosis and easier to inhibit fixation in larvae of *B. turrita* than in those of *B. flabellata*. These differences may be correlated with the longer natatory period of *B. turrita* in natural sea water. It is difficult to determine, however, whether these differences are actually specific or whether they can be attributed to altered environmental conditions that prevailed during the two summers when each type was used almost exclusively.

TABLE I

The effects of x-rays (18,333 r) and of irradiated sea water on the rate of attachment of larvae of B. flabellata. The larvae were irradiated thirty minutes after the adult colonies had been exposed to light and the irradiated sea water was seeded with larvae at the same time. The number of attached organisms in the three groups (x-rayed larvae, larvae in irradiated sea water and those in natural sea water) was counted thirty minutes later

X-rayed larvae				Larvae in irradiated sea water			Control larvae		
No. of exp.	No. of larvae	No. attached	Per cent attached	No. of larvae	No. attached	Per cent attached	No. of larvae	No. attached	Per cent attached
1	104	87	84	87	85	98	100	50	50
2	214	207	97	66	63	95	63	62	98
3	46	44	96	191	190	99	145	48	33
4	150	143	95	—	—	—	349	183	52
5	248	242	98	—	—	—	247	158	64
6	340	330	97	—	—	—	257	130	50
7	170	158	93	—	—	—	167	55	33
8	151	141	93	—	—	—	94	20	21
	Average per cent		94 ± 4						50 ± 24

The *t* ratio for the significance of the difference of the means of the x-rayed and control larvae = 5.12; $P = < .005$. The *t* ratio was computed from percentages carried out to one decimal point (not the rounded percentages shown in columns 4 and 10).

Although either x-raying larvae of *B. turrita* or seeding them in irradiated sea water induced more rapid attachment of the organisms, subsequent development was seriously impeded. Frequently larvae that had been x-rayed failed to develop after attachment. In other cases undifferentiated growth occurred at a much retarded rate. Instead of forming normal zoids, the larvae merely developed elongated masses of clear, gelatinous, stolon-like material without any internal organization.

Usually these growths formed at opposite sides of the body of the larva. One growth evidently corresponded to the stolon for attachment of the organism and the other developed in the region where the body of a normal zoid usually forms. Both growths were abnormally long and slender. The material corresponding to the stolon of a normal zoid rarely differentiated into the four knob-like projections, symmetrically placed and each branching dichotomously, which are characteristic of stolons of *B. turrita.* (Stolons of *B. flabellata* have three rather than four parts.) The material which grew out from the region where the zooecium normally forms did not differentiate into a gut and tentacles.

TABLE II

The effect of x-rays (15,733 r) and of irradiated sea water on the rate of attachment of larvae of B. turrita. The larvae were irradiated thirty minutes after the adult colonies had been exposed to light and the irradiated sea water was seeded with larvae at the same time. The number of attached organisms in the three groups (x-rayed larvae, larvae in irradiated sea water and those in natural sea water) was counted eight hours later

Temp. = 24–26° C.

X-rayed larvae				Larvae in irradiated sea water			Control larvae		
No. of exp.	No. of larvae	No. attached	Per cent attached	No. of larvae	No. attached	Per cent attached	No. of larvae	No. attached	Per cent attached
1	102	100	99	75	65	87	36	12	33
2	60	56	93	194	192	99	48	38	79
3	26	23	88	140	130	93	31	17	55
4	41	35	85	102	101	99	46	28	61
5	15	14	93	56	48	86	26	16	62
6	45	36	80	131	76	58	43	15	35
7	32	28	88	180	154	85	28	20	71
8	57	43	75	96	65	88	47	19	40
9	49	43	88	78	69	88	50	20	40
10	24	21	88	20	10	50	22	15	68
11	45	40	89	23	14	61	55	22	40
12	50	45	90	24	19	79	41	29	71
	Average per cent		88 ± 6			81 ± 16			54 ± 16

The *t* ratio for the significance of the difference of the means of the x-rayed and control larvae = 6.64; P = <.001. The *t* ratio for larvae in irradiated sea water vs. the controls = 4.09; P = .005. The *t* ratios were computed by using the rounded percentages in columns 4, 7, and 10.

Larvae that attached in irradiated sea water developed similar undifferentiated growths. These zoids, however, elongated more than those formed from larvae that had been x-rayed. In fact, growth sometimes exceeded that of the controls, but the transparent gelatinous material, often peculiarly twisted, lacked internal organization. Larvae that attached to the surface film sometimes developed normal stolons. Those that attached to the bottom frequently formed long slender stolons, about three times normal length, with a spherical mass in the region where they were attached to the substrate; and some were attached by two stolons. A few developed a bud in the zooecial wall for a second zoid. But even when the original irradiated sea water had been replaced several times by fresh sea water, the growth corresponding to the zooecium failed to differentiate a gut and tentacles. Only a single larva formed a well-developed zoid with everted tentacles; and this differentiation occurred only after six days, whereas internal organization can readily be detected in a normal zoid by the end of forty-eight hours. Thus, a notable feature of larvae that were either x-rayed or placed in irradiated sea water was growth without differentiation; and the development of x-rayed larvae was more drastically impeded than that of organisms seeded in irradiated sea water.

These observations seem to be reasonably consistent. Nevertheless, it would be premature to ascribe the failure of the zoids to differentiate in a normal manner solely to ionizing radiations. X-raying the larvae not only markedly reduces their motility but also causes them to settle on the bottom of the container, and larvae which attach geopositively usually do not develop as well as those which affix themselves to the surface film. Factors affecting larval differentiation are at present largely unknown. And judgments concerning degrees of growth are more subjective than those based on numerical data. In an almost unexplored field of larval differentiation, experimental designs that appear to have only one variable may be deceptive unless similar replications can be obtained during two different summers and with more than one species. Since the chief purpose of the experiments was to determine the effects of x-rays and irradiated sea water on the attachment of the larvae, it would be inadvisable to draw definitive conclusions concerning the influence of these agents on development until further observations have been made.

A few experiments were performed to determine what role the surface of the plastic containers might play in attachment of the larvae. Both x-rayed larvae and organisms irradiated in sea water were emptied from the plastic containers into Stender dishes and the latter were also used for the controls. Although the larvae attached somewhat less readily in the Stender dishes, there was more rapid fixation of both x-rayed larvae and those in irradiated sea water than in the controls. It is not unlikely that some of the great variability in the time of attachment of the controls, always a puzzling situation that occurs yearly in almost every experiment, can be attributed to differences in roughness of the various Stender dishes used. The temperature of the water in which the adult colonies are kept also seems to cause variability in the time of setting (*cf.* Lynch, 1955b).

II. Oxidizing agents

One of the problems encountered in determining the possible effects of oxidizing agents on the rate of fixation of the larvae was that of getting solutions dilute enough to prevent cytolysis. Both strong and weak solutions greatly reduced the motility of the larvae. But in solutions that were too strong the larvae merely became immobilized on the bottoms of the containers without attaching themselves; and these organisms eventually cytolyzed. With solutions that were weaker the time of counting was an important factor. If counts were made too soon, no observable differences in control and experimental larvae could be detected. If counts were made too late, when the controls had metamorphosed in large numbers, again no differences could be observed. After a wide variety of concentrations had been tested, the right dilution for inducing attachment was eventually found.

Hydrogen peroxide. Table III shows that by twenty-one hours the number of attached larvae (*Bugula turrita*) in sea water containing 1:14,000 parts of 30% H_2O_2 was significantly greater than that of the controls ($P = .001$). Since there were no notable differences in the number of attached organisms in the control and experimental dishes by twelve hours, H_2O_2 had a delayed action in inducing fixation, somewhat similar to that observed after one-minute exposures of bryozoan larvae to urea (Lynch, 1957). The cause of this delayed action is unknown.

Zoids formed from larvae exposed to sea water containing H_2O_2 resembled those in irradiated sea water. Elongation sometimes exceeded that of the controls, but the zoids were usually misshapen. The larvae generally attached in greater numbers to the surface film than to the bottoms of the containers and the majority of these formed normal tetrapod stolons with dichotomous branches at their ends, but the zooecial region failed to differentiate internal organs. Geopositive larvae produced much gelatinous, stolon-like material without differentiation. None of the organisms observed during a period of a week developed tentacles, although these normally form by about forty-eight hours. Larvae of *B. flabellata,* observed during the previous summer, were less adversely affected than those of *B. turrita.* Although H_2O_2 considerably reduced the motility of the larvae, it apparently was not excessively injurious to the cilia; otherwise the organisms would have dropped to the bottoms of the containers and remained there.

Table III

The effects of 30% H_2O_2 (1:14,000 parts by volume = 0.0072 volume per cent) in sea water on the rate of attachment of larvae of B. turrita. The experimental and control solutions were seeded with larvae thirty minutes after the adult colonies had been exposed to light (except no. 1, which was seeded at fifty minutes). The number of attached organisms was counted twenty-one hours later. The pH was that of sea water

No. of exp.	Experimental larvae			Control larvae		
	No. of larvae	No. attached	Per cent attached	No. of larvae	No. attached	Per cent attached
1	28	28	100	23	12	52
2	62	54	87	32	15	47
3	35	34	97	72	38	53
4	16	16	100	13	6	46
5	31	31	100	17	11	65
6	20	20	100	20	18	90
7	37	36	97	43	20	46
8	35	35	100	44	22	50
	Average per cent		98 ± 2			56 ± 15

The *t* ratio for the significance of the difference of the means of experimental and control larvae (computed by using fractional percentages) = 7.07; P = <.001

The effects of TTC. Table IV shows that sea water solutions of TTC in concentrations of 1×10^{-5} *M* (pH = 7.9–8.0) also induced more rapid fixation of the experimental larvae (*B. turrita*) than that which occurred in the controls. In solutions of 5×10^{-5} *M* the larvae attached more readily than in the weaker medium,

TABLE IV

The effect of 0.00001 M 2,3,5-triphenyltetrazolium chloride in sea water (pH = 7.9–8.0) on the rate of attachment of larvae of B. turrita. Control and experimental dishes were seeded with larvae thirty minutes after the adult colonies had been exposed to light and the number of attached organisms in each group was counted at the end of eight hours

No. of exp.	Experimental larvae			Control larvae		
	No. of larvae	No. attached	Per cent attached	No. of larvae	No. attached	Per cent attached
1	61	49	80	33	17	52
2	36	24	67	35	11	31
3	41	19	46	27	8	30
4	55	35	64	23	8	35
5	50	42	84	37	17	46
6	30	26	87	56	38	68
7	44	41	93	58	34	59
	Average per cent		74 ± 15			46 ± 16

The *t* ratio for the significance of the difference of the means of the control and experimental larvae (computed by using fractional percentages) = 3.47; P = .015

but subsequent development was considerably retarded. If the Stender dishes were flooded with fresh sea water after the larvae had attached, growth took place at a markedly reduced rate after exposure to either concentration of TTC. Some of these organisms formed tentacles. Larvae left in the weaker of the two TTC

TABLE V

The effect of sodium 2, 6-dichlorobenzenoneindophenol in concentrations of 0.01 mg./liter of sea water (3.4×10^{-8} M) on the rate of attachment of larvae of B. turrita. The experimental and control Stender dishes were seeded with larvae thirty minutes after the adult colonies had been exposed to light and the number of attached organisms was counted seven hours later. Temp. = 24–26° C. The pH of the experimental solution was that of sea water

No. of exp.	Experimental larvae			Control larvae		
	No. of larvae	No. attached	Per cent attached	No. of larvae	No. attached	Per cent attached
1	56	50	89	42	24	57
2	81	59	73	53	16	30
3	64	31	48	35	11	31
4	49	42	86	115	28	24
5	82	46	56	97	55	57
6	128	92	72	96	47	49
7	82	53	65	197	132	67
8	205	127	61	61	36	59
9	58	44	76	64	34	53
10	18	17	94	44	27	61
	Average per cent		72 ± 15			49 ± 17

The *t* ratio for the significance of the difference of the means of the experimental and control larvae (obtained by using rounded percentages in columns 4 and 7) = 3.08; P = .015.

solutions elongated slightly by twenty-four hours and these zoids resembled those in sea water containing H_2O_2 insofar as there was an abnormal amount of gelatinous material without differentiation; organisms left in the stronger solution did not develop. Larvae that attached in solutions of 5×10^{-5} *M* TTC became slightly pink, indicating a reduction of the solution; those in the weaker solution became faintly pink. Although TTC solutions exposed to air turned somewhat pink by eight hours, either with or without organisms in them, the larvae were always more deeply colored than the solutions.

The effects of SDIB. This oxidizing agent, while inducing fixation of the larvae at a rate significantly higher than that of the controls ($P = .015$), as shown in Table V, was less injurious to the larvae than any of the other agents discussed in this paper. Larvae that were left in solutions of 0.01 mg./liter of sea water ($pH = 7.9$) generally developed normally but at a rate somewhat slower than the controls. A preponderance of settings occurred at the surface film, and these developed better than those on the bottom. Larvae that attached to the bottoms of the Stender dishes formed zoids without any differentiation by forty-eight hours. But development was variable in these solutions, sometimes equalling that of the controls and sometimes being inferior.

III. Reducing agents

A very limited number of preliminary experiments was carried out with two reducing agents, sodium bisulfite and sodium thiosulfate, to determine whether their action on attachment would be opposite to that of oxidants. But neither reducing agent prevented attachment. Sea water solutions of sodium thiosulfate as strong as 10 mg./liter and concentrations of sodium bisulfite of 0.0001 *M,* 0.001 *M* and 0.005 *M* did not prevent attachment and metamorphosis. Nor did these solutions appear to have any inhibitory effects on the larvae. Although it seems unlikely that further experimentation with these solutions will show that they inhibit attachment, a greater variety of concentrations should be tested before a definitive conclusion can be reached concerning their action.

DISCUSSION

Since the action of x-rays in inducing fixation of *Bugula* larvae can be simulated by irradiated sea water, the effect on attachment appears to be an indirect one. Other instances of a similar indirect effect of ionizing radiations in living material have been reported in the literature. Barron and his co-workers, for instance, believed that the inhibiting effect of x-rayed sea water on the respiration of sea urchin sperm was attributable to stable organic peroxides formed during irradiation of the medium (Barron *et al.,* 1949). Similarly, Wichterman and Figge (1954) found that when paramecia were x-rayed the lethality of the radiations was correlated with the extent of exposure to air of the culture medium. These investigators concluded that the lethal factor was probably H_2O_2 or some other oxidation product formed in the moist air surrounding the culture fluid; their paper contains a brief review of the literature. The apparently indirect action of x-rays in inducing fixation of bryozoan larvae may offer a possible explanation of the seemingly strange observation of Bertholf and Mast (1944) that extracts of muscle tissue from rabbits killed with x-rays had an accelerating effect on ascidian metamorphosis.

The action of hydrogen peroxide and the other oxidizing agents discussed in this paper poses an interesting question concerning the effects of four other agents in inducing metamorphosis: copper, basic dyes, iodine and quinone (Lynch, 1949, 1952, 1956, 1957). These substances are also inhibitors of the dehydrogenases, and the action of some of them can be reversed by cysteine (references for copper and iodine are given by Needham, 1950, p. 425; for quinone see White *et al.,* 1954; and for basic dyes *cf.* Quastel and Wheatley, 1931). The difference in effect on attachment produced by cysteine on the one hand (Lynch, 1957) and by iodine, copper and the basic dyes on the other, might be an indication that the latter inactivate an enzyme system, such as succinic dehydrogenase, and that this inactivation abruptly ends larval life allowing the adult action system to gain control. Such a hypothesis has been proposed by Glaser and Anslow (1949) as a possible explanation of the action of copper in inducing metamorphosis in ascidian tadpoles; these investigators have suggested that copper may operate alternately as an electron donor or acceptor in the prosthetic group of some oxidizing enzyme, which they visualize as being a porphyrin-like ring compound. The data on bryozoan metamorphosis do not preclude the possibility that oxidizing enzymes may be involved, but it seems unlikely that succinic dehydrogenase plays an important role in the fixation of *Bugula* larvae, for this enzyme is inhibited by urethane (White *et al.*, 1954). If the effect were primarily on this enzyme system, one would expect urethane to act like quinone, copper, iodine and the basic dyes; but urethane inhibits fixation, whereas the other four agents induce attachment (Lynch, 1957).

The effect of these agents on succinic dehydrogenase in other organisms, however, may give a clue concerning their action on the colloidal state of protoplasm. It has long been suspected that oxidizing and reducing agents play an important part in blood clotting (Matthews, 1936). And it has been suggested that the conversion of fibrinogen into fibrin involves an oxidation process. Chargaff and Bendich (1943) believe that this oxidation involves aminoacyl groups of proteins; Baumberger (1941), on the other hand, considers that the oxidation of sulphydryl groups is of prime importance in the clotting mechanism. And the significance of certain reducing agents which inhibit the conversion of prothrombin into thrombin has been emphasized by Carter and Warner (1954). Recently Mazia and Dan (1952) have shown that the spindle fibers of cells undergoing mitosis can be isolated by creating artificial disulfide bonds when these cells are treated with H_2O_2 (or iodine) before the rest of the cell content is solubilized by a detergent. These investigators believe that H_2O_2 removes hydrogen from the sulphydryl groups of proteins and converts these substances into less soluble material by joining them together by —S—S— bonds. Thus there appears to be a polymerization of smaller molecules through these disulfide bridges. Calcutt (1951), likewise, thinks that certain photodynamic dyes affect the exposed —SH groups of the protein molecule.

The diversity of factors capable of inducing attachment of bryzoan larvae seems to indicate that these agents have a direct effect on the fluid of attachment. In many of the sessile organisms the cementing substance appears to be a mucoprotein (*cf.* Pyefinch and Downing, 1949), and Knight-Jones (1953) has found evidence that barnacles attach by means of a substance which he considered to be a quinonetanned protein, a compound similar to the material in the hardened cuticle of an insect. The action of x-rays in inducing fixation of *Bugula* larvae would not be out of harmony with the working hypothesis that attachment, when artificially induced, is brought about by agents which cause coagulation. Such a coagulating effect of x-rays has been reported for such varied types of protoplasm as sea urchin eggs (Rieser, 1955) and paramecia (Wichterman and Figge, 1954). On the other hand, irradiation of fibrinogen prolongs the clotting time (Rieser, 1956). The possibilities just discussed may form a link which would connect the effect of photodynamic dyes with that of x-rays, since both agents may release H_2O_2 or organic peroxides (Blum, 1941, p. 96; Barron *et al.*, 1949). It would be reasonable to suspect that the action of both iodine and quinone would be similar to that of H_2O_2.

The excellent development of zoids formed from larvae whose metamorphosis had been induced by treatment with sea water containing neutral red in parts of 1:100,000 (Lynch, 1952) and the poor development of zoids in solutions of H_2O_2 may be attributed, perhaps, either to unrecognized extrinsic factors affecting the latter or to the higher concentration of H_2O_2 (1:14,000 parts). Experiments with concentrations of neutral red that were ten times stronger than those used for the observations previously reported showed that larvae in these media also failed to develop after attachment.

SUMMARY

1. X-raying larvae of either *B. flabellata* (18,333 r) or *B. turrita* (15,733 r) within thirty minutes after the organisms began to emerge from the parental colonies induced more rapid setting than that which occurred in the controls ($P = .005$ and .001, respectively). Irradiated sea water had a similar, but slightly less pronounced, effect ($P = .005$). In these experiments the subsequent development of larvae of *B. turrita* into zoids was drastically impeded. Slow growth, usually without differentiation, was observed.

2. Sea water solutions of H_2O_2 (7×10^{-4} *M*), of 2,3,5-triphenyltetrazolium chloride (1×10^{-5} *M*) and of sodium 2,6-dichlorobenzenoneindophenol (3.4×10^{-8} *M*) at a pH of 7.8–8.0 also induced more rapid setting of the experimental larvae ($P = .001$, .015 and .015, respectively). The subsequent development of larvae exposed to H_2O_2 and to 2,3,5-triphenyltetrazolium chloride resembled that of organisms that were either x-rayed or placed in irradiated sea water. Sodium 2,6-dichlorobenzenoneindophenol was less injurious to the larvae than the other agents used. An explanation of the possible role of these agents in inducing an accelerated rate of setting is presented.

LITERATURE CITED

BARRON, E. S. G., VERONICA FLOOD AND BETTY GASVODA, 1949. Studies on the mechanism of action of ionizing radiations. V. The effect of hydrogen peroxide and of x-ray irradiated sea water on the respiration of sea urchin sperm and eggs. *Biol. Bull.*, **97**: 51–56.

BAUMBERGER, J. P., 1941. Some evidence in support of a sulphydryl mechanism of blood clotting. *Amer. J. Physiol.*, **133**: 206–207.

BERTHOLF, L. M., AND S. O. MAST, 1944. Metamorphosis in the larva of the tunicate, *Styela partita*. *Biol. Bull.*, **87**: 166.

BLUM, H. F., 1941. Photodynamic Action and Diseases Caused by Light. Reinhold Pub. Corp., New York.

CALCUTT, G., 1951. The role of radiation in photodynamic action. *J. Exp. Biol.*, **28**: 537–541.

CARTER, J. R., AND E. D. WARNER, 1954. Evaluation of disulfide bonds and sulphydryl groups in the blood clotting mechanism. *Amer. J. Physiol.*, **179**: 549–556.

CHARGAFF, ERWIN, AND AARON BENDICH, 1943. On the coagulation of fibrinogen. *J. Biol. Chem.*, **149**: 93–111.

EVANS, T. C., 1947. Effects of hydrogen peroxide produced in the medium by radiation on spermatozoa of *Arbacia punctulata*. *Biol. Bull.*, **92**: 99–109.

GLASER, OTTO, AND GLADYS ANSLOW, 1949. Copper and ascidian metamorphosis. *J. Exp. Zool.*, **111**: 117–140.

KIMBALL, R. F., AND N. GAITHER, 1952. Role of externally produced hydrogen peroxide in damage to *Paramecium aurelia* by x-rays. *Proc. Soc. Exp. Biol. Med.*, **80**: 525–529.

KNIGHT-JONES, E. W., 1953. Laboratory experiments on gregariousness during setting in *Balanus balanoides* and other barnacles. *J. Exp. Biol.*, **30**: 584–598.

LYNCH, W. F., 1949. Acceleration and retardation of the onset of metamorphosis of two species of Bugula larvae from the Woods Hole region. *J. Exp. Zool.*, **111**: 27–55.

LYNCH, W. F., 1952. Factors influencing metamorphosis of Bugula larvae. *Biol. Bull.*, **103**: 369–383.

LYNCH, W. F., 1955a. Synergism and antagonism in the induction of metamorphosis of Bugula larvae by neutral red dye. *Biol. Bull.*, **109**: 82–98.

LYNCH, W. F., 1955b. Phototropic responses of Bugula larvae in the presence of stimulating and anaesthetic agents. *Proc. Iowa Acad. Sci.*, **62**: 652–662.

LYNCH, W. F., 1956. Experimental modification of the rate of metamorphosis of Bugula larvae. *J. Exp. Zool.*, **133**: 589–612.

LYNCH, W. F., 1957. The effects of certain organic compounds and of antimitotic agents on the rate of metamorphosis of Bugula and Amaroecium larvae. *J. Exp. Zool.*, in press.

MATTHEWS, A. P., 1936. Principles of Biochemistry. Wood, Baltimore.

MAZIA, DANIEL, AND KATSUMA DAN, 1952. The isolation and biochemical characterization of the mitotic apparatus of dividing cells. *Proc. Nat. Acad. Sci.*, **38**: 826–838.

NEEDHAM, JOSEPH, 1950. Biochemistry and Morphogenesis. Cambridge at the University Press.

OKA, SHUZITU, 1954. Radiosensitivity in *Lophopodella carteri*, a fresh water bryozoan. *Sci. Rep. Tokyo Bunrika Daigaku*, **111**: 211–217.

PYEFINCH, K. A., AND S. F. DOWNING, 1949. Notes on the general biology of *Tubularia larynx* Ellis and Solander. *J. Mar. Biol. Assoc.*, **28**: 21–43.

QUASTEL, J. H., AND H. M. WHEATLEY, 1931. The action of dyestuffs on enzymes. *Biochem. J.*, **85**: 629–639.

RIESER, PETER, 1955. The effect of roentgen rays on the colloidal properties of the starfish egg. *Biol. Bull.*, **109**: 108–112.

RIESER, PETER, 1956. Radiation-induced alteration of fibrinogen clotting rate and clot lability. *Proc. Soc. Exp. Biol. Med.*, **91**: 654–657.

WHITE, ABRAHAM, PHILIP HANDLER, E. L. SMITH AND DEWITT STETTEN, 1954. Principles of Biochemistry. McGraw-Hill Book Co., Inc., New York.

WICHTERMAN, RALPH, AND F. H. J. FIGGE, 1954. Lethality and the biological effects of x-rays in Paramecium; radiation resistance and its variability. *Biol. Bull.*, **106**: 253–263.

GASTRULAR BLOCKAGE IN FROGS' EGGS PRODUCED BY OXYGEN POISONING[1]

SASHA MALAMED[2, 3]

Department of Zoology, Columbia University,
New York 27, N. Y.

Seldom do embryos stop developing without dying. Occasionally, however, maintenance becomes temporarily independent of gross morphological change as in the diapause of insects. Although non-reversible, this condition may be experimentally produced in amphibians by a few methods which result in highly uniform populations of arrested embryos which stay alive, that is, do not cytolyze for a relatively long time. Well-known among these techniques are CN^- or azide treatment (Spiegelman and Moog, 1945), and certain hybridizations (Moore, 1941; Brachet, 1944). In the late forties, however, Nelsen (1947, 1948, 1949, 1950) obtained a gastrular block in frogs' eggs by using 3 atmospheres of oxygen added to air at standard pressure. This method offers certain advantages toward a causal analysis of development.

First, the agent's effect is not immediate as in the case of azide, nor is it necessary to treat the embryos continuously as with CN^-. After 24 hours of treatment with oxygen pressure, the embryos are in the early cleavage stages. Until late blastulation they cannot be distinguished from the controls. Development stops just before dorsal lip formation and cytolysis does not set in for at least 30 hours. Embryos are thus obtainable with chemical aberrations which have not yet appeared at the morphological level.

Second, while each of the other methods except that employing azide has an all-or-none effect, the influence of oxygen pressure may be varied by controlling the dosage. By reducing the latter, "incompletely blocked" embryos are produced. These develop into larvae of normal appearance except for the scar of an abnormal gastrulation in the form of a persistent yolk plug.

Third, azide will affect any pre-gastrular stage. Although CN^- and hybridization have effects which are largely specific for gastrulation, oxygen pressure's specificity is even sharper. Clayton (1950) has shown in embryos pre-treated with the appropriate dose of oxygen that the movements of the notochord anlage are not prevented, though some of the other gastrular movements cease. Thus, incompletely blocked embryos do form neural tubes. With either CN^- or hybridization, however, gastrulation is completely blocked and all the movements stop.

Before exploiting these advantages afforded by the oxygen pressure effect, certain preliminary problems needed clarification. Accordingly, the present report is an investigation of 1) whether it is oxygen or pressure or both that affects the embryos, 2) dosage requirements, and 3) whether there is any effect on developmental rate prior to the gastrular block as in the case of CN^-. Although the latter does not stop pre-gastrular development, the fact that it slows these stages shows that it attacks a system on which development is dependent before as well as during gastrulation and thus detracts from its value as an analytical tool for the study of chemical events peculiar to the process of gastrulation.

---

[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Faculty of Pure Science, of Columbia University.

[2] Public Health Service (Predoctorate) Research Fellow of the National Cancer Institute.

[3] Present address: Department of Physiology, Western Reserve University School of Medicine, Cleveland 6, Ohio.

MATERIALS AND METHODS

Eggs of *Rana pipiens* from Vermont were obtained by the pituitary injection method of Rugh (1948, pp. 102–106) and fertilized by his method with slight modifications. After injection the females were kept at 12° to 14° C. until stripped; insemination was at room temperature. Fertilization and development of the embryos was in 10% frog Ringer's solution.

A few experiments were performed shortly after the natural breeding season of *R. pipiens.* For this purpose gravid females were stored at 5° C. from January until used as above. These "summer" frogs were kept in a 0.03% tap water solution of sodium sulfadiazine until injection.

Stages of development were designated according to Shumway (1940) and with reference to Rugh (1948, pp. 63–65). The designated stage was the latest one which at least one-half the embryos had reached.

APPARATUS

An apparatus was constructed consisting of 6 glass pressure chambers which were shaken and kept at constant temperature. The chambers were continuous or discontinuous with each other in various combinations. Thus 6 levels of either of 2 variables could be studied at the same time, that is, on samples of a single clutch of eggs. These dosage variables were pressure and hours of treatment. In addition it was possible to have 3 chambers stationary with simultaneous shaking of the others. This permitted the effect of shaking to be determined on one clutch of embryos at 3 levels of either of the 2 dosage variables. Since the apparatus included only one water bath, comparative temperature studies on single clutches were precluded. Provision was made for the inclusion of 4 control bottles, shaken and unshaken, and containing eggs under no increased oxygen pressure.

The pressure manifold with its 3 gauges was mounted above a rectangular Warburg apparatus (Fig. 1). The latter provided the temperature regulation and shaking mechanism needed. Rubber pressure tubing connected the manifold to the pressure chamber assemblies submerged in the water bath. On each bank of the Warburg apparatus were mounted a few manometer supports connected by means of an aluminum rod fastened to their horizontal arms. Shaking of the pressure chambers was effected by clamping the assemblies to this rod.

The pressure chamber assemblies (Fig. 2) were slightly modified units of the Parr hydrogenation apparatus. In each assembly, a 500-ml. Pyrex glass bottle (surrounded by a perforated steel shield) served as the pressure chamber for the embryos.

The pressure manifold (Fig. 1) was assembled of ¼-inch galvanized iron pipe and brass fittings. The nozzles of the manifold and of the pressure chamber inlet

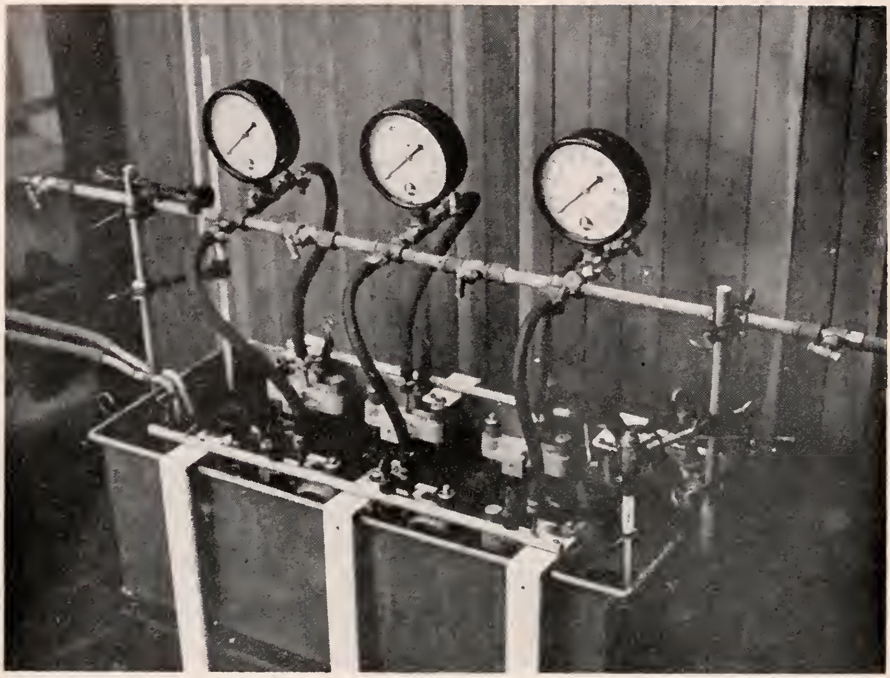


FIGURE 1. Constant-temperature pressure apparatus with provision for shaking.

tube permitted a flexible connection of rubber pressure tubing and this, in turn, allowed for shaking of the pressure chamber assemblies while the manifold was stationary. The gauges supplied by the Parr Instrument Co. were of the Bourdon type and read from 0 to 100 p.s.i. (pounds per square inch) in units of 1 which were large enough for estimation of the needle position to the nearest 0.2 p.s.i.

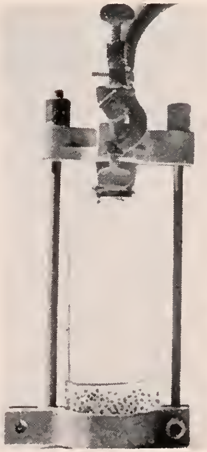


FIGURE 2. Pressure chamber assembly.

They had a specified accuracy of at least ± 0.5 p.s.i. When in a closed system, with gas supplied from a single source, the gauge readings up to 50 p.s.i. agreed to 0.2 p.s.i. The 10 valves with stainless steel needles were from Hoke, Inc. A commercial pressure cylinder supplied the gas which entered the manifold through either terminal valve; by shutting the other one, a closed system was effected. To decompress the first pressure chamber in a time series the appropriate terminal valve was opened. For the others in the series, non-terminal valves were used. The specified purity of the gases obtained from the Ohio Chemical and Surgical Equipment Co. and from the Matheson Co. was 99.5%. The principal impurity of the oxygen was nitrogen and vice versa. For each experimental run, the settings of the needle valves were determined by the variables to be studied.

The system permitted positive pressures up to 50 p.s.i. The total leakage during the course of any run never exceeded 5% of the gauge readings registered at the start. Actually, more than 2% leakage seldom occurred.

Temperature control was maintained as in ordinary procedures with the Warburg apparatus, with one exception. Since all the runs were below room temperature, cooling coils were added to the floor of the water bath. Through these coils flowed water which was cooled in a separate water bath by a portable refrigerator. This "cooling bath" was temperature-controlled at about 5° C. below the temperature desired in the Warburg bath. The heating unit of the latter operated intermittently against this continuous cooling. The Warburg bath was run at 18.0°, 12.0°, or 8.0° C. with a variability of ± 0.05° C. in each case.

Shaking was at a rate consistent with normal development of the embryos and rapid gas diffusion between the liquid phase containing the eggs and the gas phase above it. The stationary surface area was 4.9 square inches and 50 ml. of 10% Ringer's solution were used. The depth in the pressure bottle of the 10% Ringer's solution plus the embryos was 1 inch. The Warburg was altered to permit shaking on each bank at 30 ± ½ c.p.m. in a horizontal plane with amplitude of 1½ inches. In a few runs with a preliminary apparatus, the shaking rate was 36 ± 1 c.p.m.

Procedure

Each experimental run may be generally divided into 3 phases: 1) fertilization and compression, 2) decompression and selection of embryos, and 3) tabulation of abnormalities. Table I summarizes these steps.

1) At room temperature and 30–45 minutes after insemination, the clutch of eggs is rinsed with 10% Ringer's solution and then cut up into groups of 20–40 eggs. Only those clutches of eggs in which at least 80% rotate are used. The animals are distributed about 300 to a pressure bottle, each of the latter containing 50 ml. of 10% Ringer's solution. Including one control, 7 bottles are usually loaded. The metal-shielded bottles are fitted to the rubber stoppers attached to the manifold of the apparatus, then the clamps to the bottles. The former are tightened and, with the addition of the control bottle(s), are attached to the aluminum rods, thus submerging them all in the water bath, the latter at 18.0°, 12.0°, or 8.0° C. Now shaking is begun and pressure is built up gradually and simultaneously in all the bottles over a period of 20 minutes. The midpoint of this period is noted as the start of pressure treatment. The bottles are not flushed; the oxygen is added to the air in them; hence the total pressure in each bottle

equals the sum of the gauge reading for oxygen plus 1 atmosphere of air. After application of pressure the remaining eggs are observed to make sure that the first cleavage has not yet occurred. The bottles are shaken continuously until the time for decompression except in certain experiments where the effect of shaking is studied.

2) Decompression is gradual over one hour, and the midpoint of this period of time is taken as the end of pressure treatment. The order and times of decompression of the individual chambers in any one run varies, of course, with the purpose of the latter. While pressure is being released, work is progressively begun on the contents of each bottle already decompressed and immediately unloaded. The contents of the bottles containing embryos plus medium still at a temperature near that of the water bath are emptied into a finger bowl containing about 50 ml. of fresh 10% Ringer's solution at room temperature. From this finger bowl, when they are at the mid- or late blastula stage, 100 (or, in a few experiments, 50) apparently normal eggs are selected. If, after decompression, less than 80% of the embryos appear normal in either the control or treated samples, no embryos are selected and the run is discontinued. In the selection process, the blastulae are transferred to a second finger bowl containing fresh 10% Ringer's solution. After staging of the embryos, all the finger bowls are placed at 18.0° C.

TABLE I

Summary of procedure

Phases	Steps	Temperature changes of eggs	Relative time approximately[Δ]		Stage of development of normal eggs
1.	Bath on				
	Fertilization	Room temp.	0 min.		
	Rotation		45 min.		
	Loading	Bath temp.	45 min.–100 min.		
	Shaker on				
	Compression		100 min.–120 min.		Before 3
			First	Last	
			Bottle		
2.	Decompression		11–12 hrs.	16–17 hrs.	
	Unloading	Room temp.			
	Fresh Ringer's			*(A)	Cleavage 8, 9
	Selection (Fresh Ringer's)				
				*(B)	
		18° C.	13 hrs.	19 hrs.	
3.	Tabulation	Room temp.	71–74 hrs.		14

* Staging of controls and last treated sample to be decompressed for developmental rate studies.

[Δ] For relative times of controls follow sub-column for last bottle, and disregard times for compression and decompression.

This results in a set of finger bowls, each of which corresponds to one of the pressure bottles. The 100 eggs in each appear normal and are in a uniform stage of development earlier than gastrulation. These embryos are left to develop at 18.0° C. until neural fold formation of the controls.

Because of the selection process, pre-gastrular abnormalities are eliminated from the populations to be counted at the end of each run. Actually, oxygen treatment and shaking, either singly or in combination, are found by comparison with control groups of eggs to have little if any effect on development prior to gastrulation. The great majority of unhealthy eggs selected out are unfertilized. The selection process is completed before gastrulation.

Selection (as well as each of the other steps) is the same for the controls as for the treated eggs. The former are removed from the Warburg bath with the last of the series to be decompressed. Thus each member of these pairs of oxygen-treated and untreated embryos will have had the same history of temperature environments at the time of neurulation when abnormalities are counted. Collation of "stagings" on these sets from different runs constitutes the developmental rate studies.

3) When the control eggs are neurulas, cytolysis has not yet occurred in the abnormal embryos. At this time all the finger bowls are transferred to room temperature and the numbers of normal and neurulating embryos in each finger bowl are counted. If less than 95% of the controls are normal, the run is disregarded. In the abnormal class, evidence of developmental arrest or abnormality before stage 9 or 9 + is never found, and since only rarely does an abnormal neurula occur which does not show a gastrular aberration, that is, unincorporated yolk, the results are expressed as numbers or per cents of normal gastrulae.

Results

1. Identification of the Effective Agent

When *Rana pipiens* embryos are treated with oxygen during early cleavage they stop developing normally at the late blastula stage, well after decompression. The columns for oxygen in Table II show typical results with various dosage conditions.

Since effective oxygen treatment involves the use of pressure, the question arose as to the role that is played by this factor. Experiments were run using nitrogen instead of oxygen. They were otherwise identical in procedure and equal or greater in dosage than the oxygen runs which invariably produce embryos which fail to gastrulate normally. In none of the nitrogen experiments did more than 3% of the embryos block at gastrulation. Table II, which includes results with oxygen for comparison, shows no mechanical effect of pressure on development.

The ineffectiveness of pressure "alone" was confirmed by another type of experiment in which one bottle of a pair was shaken and the other was not. Except for this difference, the embryos in the two bottles had the same oxygen treatment. Several pairs of bottles were used, each pair for a different duration of treatment. Table III shows that even though pressure (and here the gas was oxygen) was the same in the stationary bottles as in the shaken ones, only in the former was gastrulation normal.

It might appear from consideration of Table III that shaking is the effective factor that we seek. But control embryos in air at atmospheric pressure are routinely shaken and show better than 95% normal development, for otherwise an

Table II

Per cent normal gastrulae with comparable treatments of oxygen and nitrogen. Air at 1 atmosphere. Treatment at 8.0° C.; 100 eggs per sample except at 45 p.s.i. of oxygen with 23 hours where 50 eggs used. Nitrogen samples from same cross. Oxygen samples from different crosses. All samples shaken at 30 c.p.m.

P. S. I. added to air	45				30		
Gas	O_2	N_2	O_2	N_2	O_2	N_2	N_2
Hours of treatment	14	14	23	24	23	24	38
Per cent normal gastrulae	25	97	0	99	5	99	99

experiment is disregarded. Gastrulation is normal even when shaking is combined with nitrogen pressure (see Table II).

Since shaking and pressure treatment singly or together are ineffective, and yet the two together with oxygen produce gastrular blockage, it becomes apparent that oxygen at the given pressures is the effective agent. The reason why the given pressures are required may be explained by Dalton's Law which states that in a two-phase system the solubility of a given gas in the liquid phase is directly proportional to its partial pressure in the gas phase above the liquid. Raising the oxygen pressure to the given hyperatmospheric levels, then, is one way to increase the solubility of oxygen in the medium so that at equilibrium the oxygen concentration is at a toxic level. Shaking the system speeds saturation of the medium after oxygen pressure is built up over it.

If, after oxygen pressure is applied, shaking serves merely as an aid in bringing the toxic agent, oxygen, through the 10% Ringer's solution to the embryos, then embryos which are not shaken should also be poisoned when treated for an additional period to allow for the slow reaching of equilibrium between the gas and liquid phases. To illustrate this point two series were run, one with shaking and the other without it. In each series several durations of oxygen treatment were used, a different sample of 50 embryos for each dosage. The two curves of Figure 3 show that to produce a given number of abnormal gastrulae it took roughly 5 hours more under oxygen pressure without shaking than it did with shaking. Two control series were also run at ambient air pressure, one series with shaking, and the other without shaking. In either series, at least 48 of the 50 embryos in each sample developed normally.

Before concluding that gastrular blockage is caused by excess oxygen in the liquid environment of the embryos, two miscellaneous possibilities must be eliminated. These are 1) that the embryos suffer from shock resulting from short compression and decompression periods and 2) that they are overcrowded (see Barth, 1946). As for the first consideration, reference to Table III, Figure 3, and the data in the next section (Dosage) reveals the many short doses of oxygen treatment, including those with shaking, which did not produce abnormal gastrulae. In

Table III

Per cent normal gastrulae with oxygen treatment with and without shaking. Treatment at 18.0° C. with 45 p.s.i. of oxygen added to air at 1 atmosphere; 100 eggs per sample. All samples from same cross. Shaking at 30 c.p.m.

Hours of oxygen treatment	10		13		16	
Shaking	−	+	−	+	−	+
Per cent normal gastrulae	92	0	93	0	95	0

all these cases, just as with the longer, harmful treatments, compression and decompression were gradual over 20 and 60 minutes, respectively. In addition, the embryos were treated in the same manner with nitrogen (*cf.* Table II) and development was normal. The possibility of overcrowding is precluded by these same data for, again, normal gastrulation resulted from conditions which were just as crowded as those which produced gastrular blockage. Furthermore, even the non-shaken, non-pressure-treated embryos showed at least 95% normal gastrulae as, of course, the shaken controls did. Actually, as far as oxygen concentration in the medium was concerned, the oxygen-treated embryos were "undercrowded." Thus it appears that neither duration of pressure change nor population density affected the embryos deleteriously.

It appears, then, that gastrular blockage is effected by a pressure-produced high oxygen concentration in the liquid environment of the embryos. Indeed, as will be shown below, the percentage of normal gastrulae varies inversely with the partial pressure of oxygen at a given duration of treatment (see Figure 8). If pressure does have some effect other than via Dalton's Law, certainly it is not through an increase in the force per unit area in the mechanical sense.

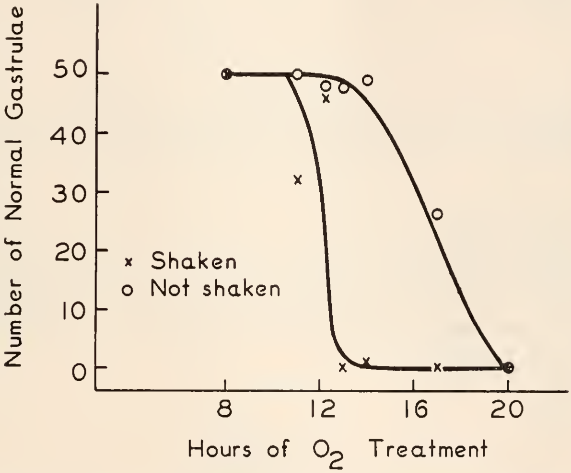


FIGURE 3. Effect of duration of oxygen treatment on gastrulation with and without shaking. Treatment at room temperature with 45 p.s.i. of oxygen added to air at 1 atmosphere; 50 eggs per sample. A different cross (no common parentage) used for each time dosage. Shaken and non-shaken samples at a given time dosage are from same cross. Shaking at 36 c.p.m.

2. Dosage

Regardless of whether duration of oxygen treatment or pressure was varied, in each series or run, pressure was applied to all the samples simultaneously and before the first cleavage. Each dosage datum obtained represented a different sample of embryos since by the time the per cent normal gastrulae of a sample was determined, the normal embryos were too advanced to be blocked at the late

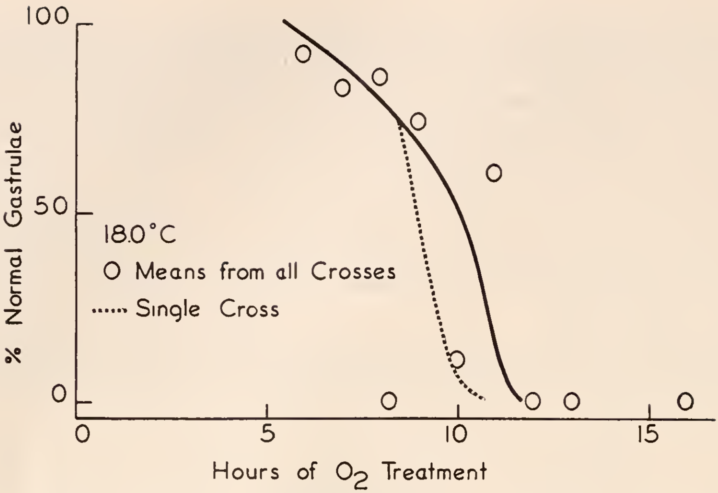


FIGURE 4. Effect of duration of oxygen treatment on gastrulation. The points represent the means from Table IV. Treatment at 18.0° C. with 45 p.s.i. of oxygen added to air at 1 atmosphere. Shaking at 30 c.p.m. Single cross from Figure 5.

blastula stage; thus a second dose on the same sample was precluded, and correspondingly, a second datum.

With few exceptions, the size of each sample was 100 embryos. Increasing a given sample to 200 made a difference of no more than 5% normal gastrulae. Occasional recounts of the same sample agreed within 2%. All the data in this section are from shaken samples.

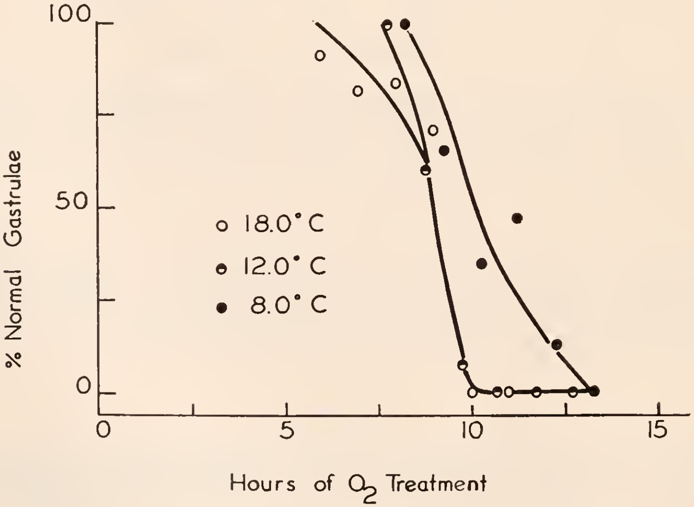


FIGURE 5. Effect of duration of oxygen treatment at various temperatures on gastrulation. Pressure is 45 p.s.i. of oxygen added to air at 1 atmosphere; 100 eggs per sample. A different cross (no common parentage) used for each temperature. Shaking at 30 c.p.m.

Curves of per cent normal gastrulae versus degrees of dosage of either kind in which all the embryos had the same parents were the most valuable, for genetic variability was thus avoided. Where, alternatively, the arithmetic averages of sets of repeat experiments were used, information was provided concerning any *R. pipiens* embryo of any parents, but the contours of the curve were softened and sharp breaks were obscured (see Figure 4).

TABLE IV

Per cent normal gastrulae with increasing durations of oxygen treatment and different parental backgrounds. Treatment at 18.0° C. with 45 p.s.i. of oxygen added to air at 1 atmosphere. Shaking at 30 c.p.m.

Exptl. run no.	Crosses	Hours of oxygen treatment									
		6	7	8	8¼	9	10	11	12	13	16
1	♀ 1 ♂ 1						0			0	0
2	♀ 2 ♂ 2		83			43					
3	♀ 3 ♂ 3	92	83	86		71	0	0			
4	♀ 4 ♂ 4					95		71			
	♀ 5 ♂ 4					90		81			
	♀ 6 ♂ 4					0		0			
5	♀ 7 ♂ 5				0		0		0		
	♀ 8 ♂ 5						0				
	♀ 9 ♂ 5						78				
	♀ 10 ♂ 5						10				
6	♀ 7 ♂ 6				0		0		0		
	♀ 8 ♂ 6				0		0				
7	♀ 11 ♂ 7					98		79			
	♀ 11 ♂ 8					96		97			
	♀ 11 ♂ 9					99		91			
Means		92	83	86	0	74	11	60	0	0	0
No. of samples		1	2	1	3	8	8	7	2	1	1

34 samples: 15 crosses: 11 ♀ ♀ (13 clutches; ♀7, ♀8 stripped twice)
100 embryos/sample 9 ♂ ♂
7 ♀ ♂ (No common parentage)

a. Duration of treatment (*time dosage and per cent abnormality*)

With increasing time dosage the per cent normal gastrulae dropped sharply. In Figure 4, a mean curve is plotted showing all the data at 18.0° C., with 45 p.s.i. of oxygen added to air at 1 atmosphere; 3400 embryos and 15 crosses are represented (see also Table IV). A curve of samples drawn from one of the 15 crosses (♀3 ♂3 in Table IV, 18.0° C. curve in Figure 5) is also presented. In either case, as soon as the embryos had been treated long enough to affect a few, it took a relatively short additional dose ("effective time dosage") to affect them all. For the curve of the means this was 6 hours.

The steepness of the slopes in Figure 4 is confirmed by Figure 5. The latter shows curves from three sets of samples, each set from a different cross with no common parents. Each set was run at a different temperature but with the same pressure dosage. It is seen that from 100% to 0% normal gastrulae took 5 hours at 8.0° C., 3 hours at 12.0° C., and 4 hours at 18.0° C. (except that in the 18.0° C. set the highest figure is only 92%). As a matter of fact these figures are maximal. If they are in error due to the points being taken at intervals of no less than an hour, correction would only shorten the effective time dosage.

Additional evidence of the shortness of the effective time dosage is provided by Figure 6 which shows the data from all crosses run at 8.0° and at 18.0° C. At each temperature the pressure dosage was the same. Each cluster of points is concentrated along the time axis.

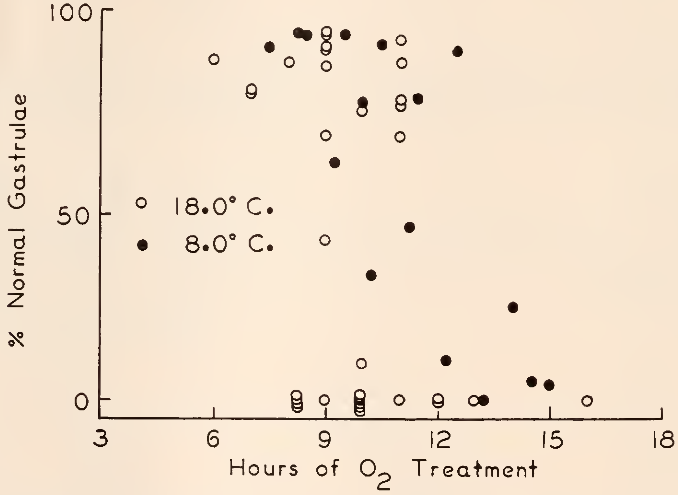


FIGURE 6. Effect of temperature on oxygen poisoning. Pressure is 45 p.s.i. of oxygen added to air at 1 atmosphere; 100 eggs per sample. Data for 18.0° C. from 15 crosses; for 8.0° C. from 5 crosses. Shaking at 30 c.p.m.

The other interesting aspect of these curves (Figs. 4, 5, 6) is the "lag dosage" before the oxygen is effective (see also Table IV). Its length of about 8 hours stands in contrast to the shortness of the effective time dosage. (Since all these data are from shaken samples, this lag is independent of that due to slow diffusion which is shown in the curve of Figure 3 for non-shaken eggs.)

b. The effects of temperature on time dosage

Oxygen solubility varies inversely with temperature. The increment in concentration of this gas is especially large from 18.0° to 8.0° C. in a saline solution (Umbreit, Burris and Stauffer, 1949, p. 5). Thus lowering the temperature has the same effect as increasing the pressure (*cf.* Dalton's Law) and this, it will be seen in a later section, decreases the duration of treatment necessary for gastrular blockage (see Figure 8).

Also lowering the time dosage is a second effect of a drop in temperature. Sensitivity to oxygen decreases with developmental age (Nelsen, 1949). Since lowered temperature also slows development, any given duration of treatment is more effective at a lower temperature than at a higher one, for that part of the time at 18.0° C. spent on later stages is expended at 8.0° C. on the earlier, more sensitive stages. Consequently, less hours of treatment are required to produce gastrular blockage at 8.0° than at 18.0° C.

In two ways, then, temperature decrease enhances the effectiveness of oxygen treatment and tends to shift to the left a curve of per cent normal gastrulae versus hours of treatment (see Figure 7). On the other hand, unless they are very atypical, the actual chemical reactions resulting from oxygen treatment are slowed by a temperature decrease (Getman and Daniels, 1943, p. 363). This third effect tends to cancel out the other two. Thus any separation along the time axis of curves at different temperatures is a net effect.

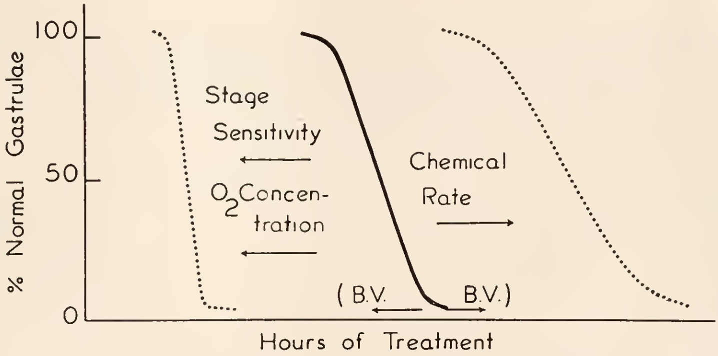


FIGURE 7. Postulated compensatory effects of decreased temperature on oxygen poisoning. B. V. stands for "Biological Variability." See text for explanation.

As far as lag dosage is concerned, neither Figure 5 nor Figure 6 shows any net effect of temperature. The former shows the results of oxygen treatment at three temperatures over a 10.0° C. range. The slight separation of the three curves is well within the variability inherent in the biological material (see Table IV) and therefore cannot be considered significant. Moreover, in Figure 6 error due to this variability is reduced through the use of samples from many crosses and here the cluster of points for 18.0° C. and those for 8.0° C. show the same lag dosage.

In Figures 5 and 6, although the lag dosage remains unaffected, the effective time dosage is increased by a temperature drop. In the former figure the slope of the 8.0° C. curve is less than that of the curves of 12.0° and 18.0° C. Although error is introduced due to the large time intervals (1 hour) between points, this error, as well as that due to biological variability, is reduced in Figure 6. Here, confirming the data of Figure 5, the cluster for 8.0° C. is more spread out along the abscissa than is the one for 18.0° C. In addition, with a fast drop in percentage as compared to a slow drop, there is smaller probability at a random time dosage of a point falling midway between 100% and 0% normal gastrulae. The points for 18.0° C. actually do aggregate at the ends of the percentage range while those for 8.0° C. are more evenly spread along the ordinate.

Thus in the range from 8.0° to 18.0° C. the several effects of temperature are fully compensatory for lag dosage, while for the effective time dosage the chemical rate effect is greater than the combination of the opposite two (see Figure 7) and a net positive temperature coefficient for oxygen poisoning is demonstrated.

c. The effect of time dosage on type of abnormality

Even though the embryos from different crosses varied greatly in their oxygen sensitivity (see Table IV and Figure 3), a rather striking uniformity of response to treatment was demonstrated among siblings. This was especially well shown when, after tabulating the per cent of normal gastrulae (class 1), the abnormal embryos in each sample of a time dosage series were broken down according to type of gastrular blockage as follows: class 2: incompletely blocked (abnormal gastrulation) and class 3: completely blocked (stage 9 or 9 +, no dorsal lip). As usual, each sample was drawn from the progeny of the same cross and represented a different time dosage; except for the latter, the conditions of treatment were kept constant.

TABLE V

Per cent of gastrulae of each class in samples exposed to increasing durations of oxygen treatment at 12.0° C. Pressure is 45 p.s.i. of oxygen added to air at 1 atmosphere; 100 eggs per sample. All samples from same cross and shaken at 30 c.p.m.

Classes of gastrulae	Hours of oxygen treatment					
	7¾	8¾	9¾	10¾	11¾	12¾
1. Normal	99	60	7	0	0	0
2. Incompletely blocked	1	40	93	63	42	1
3. Completely blocked	0	0	0	37	58	99

As is shown in Table V, with increasing time dosage each of the three classes is progressively filled, leaving always at least one null class. After class 2 reaches nearly 100% it decreases as class 3 increases. At 9¾ hours there are two null classes (1 and 3) for practically all the embryos have been treated long enough to prevent normal gastrulation, but none have yet been affected so badly as to prevent it completely. The change from 8¾ to 9¾ hours of treatment is entirely from class 1 to class 2. Those embryos already in class 2 at 8¾ hours do not enter class 3 with the additional hour of treatment. They "wait" for the embryos still normal with 8¾ hours of treatment to "catch up" and become incompletely blocked at 9¾ hours, that is, until class 2 is full before becoming completely blocked at 10¾ hours.

These data reveal two discrete thresholds of time dosage, an earlier one for incomplete blockage of gastrulation, and a later one for complete blockage. In the data of Table V, these occur, respectively, between 7¾ and 8¾, and between 9¾ and 10¾ hours of oxygen treatment.

Occasionally in runs at 8.0° C. with 45 p.s.i. of oxygen added to 1 atmosphere of air the embryos are distributed among all three classes at one time dosage. Otherwise, however, the pattern of progression from 100% normal to 100% incompletely blocked to 100% completely blocked gastrulae recurs in time dosage series run at 18.0° or 12.0° C. with 45 p.s.i. of oxygen added to air at ambient pressure or at 8.0° C. with 30 or 15 p.s.i. added to air. In addition, the same kind of results are obtained when the abnormal embryos are further subdivided into four classes.

d. Pressure dosage

In these studies, 45, 30, and 15 p.s.i. of oxygen were used; each was added to air at 1 atmosphere. Since the partial pressure of oxygen in the latter is about 0.2 atmosphere, the addition of pure oxygen in the several cases resulted in partial pressures of approximately 3.2, 2.2, and 1.2 atmospheres.

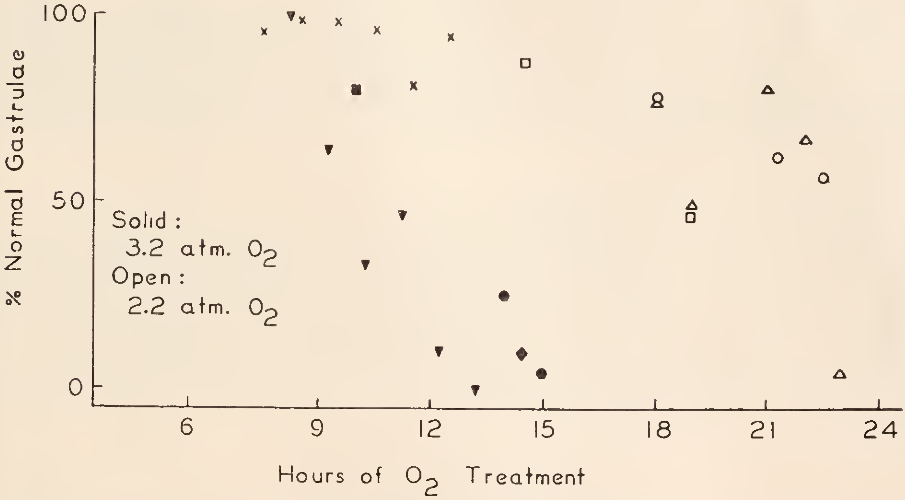


FIGURE 8. Gastrular blockage as a function of partial pressure of oxygen for various durations of treatment. Treatment at 8.0° C.; 100 eggs per sample. Samples from different crosses (no common parentage) represented by symbols of different shape. Shaking at 30 c.p.m.

Various time dosages were used with each pressure dosage except that of 1.2 atmospheres of oxygen. Each sample of embryos went through one period of treatment. The pressure was not changed during this period. Either one or several pressures of oxygen were used in a single run. All the samples in a run were from the same cross and a different cross (no common parentage) was used for each run.

Earlier, a partial pressure of 3.2 atmospheres of oxygen was found to be effective at 18.0° C. and at 12.0° C. These studies, however, were to include lower pressures and correspondingly weaker oxygen tensions in the 10% Ringer's solution. (Dalton's Law holds for oxygen to about 99% of the theoretical values in the pressure range of this work (Moore, 1950, p. 121).) In compensation, therefore, a temperature of 8.0° C. was used to ensure effectiveness of the treatment. The temperature reduction was expected (see Figure 7) to act in these ways: 1) to increase the oxygen concentration in the 10% Ringer's solution, and 2) because of decreased developmental rate, a) to concentrate the treatment on the earlier, more sensitive stages, and b) to increase the number of hours of treatment possible before gastrulation. These effects were considered of more importance than the antagonistic one of decreased chemical rate.

Figure 8 presents all the data with 3.2 and 2.2 atmospheres of oxygen plotted as per cent normal gastrulae against duration of treatment. The points fall into two separate clusters corresponding to the oxygen dosages used. An effect was also obtained with 1.2 atmospheres of oxygen. After 88 hours of treatment the embryos were in the mid-blastula stage and appeared normal. After selection at stage 9, however, none gastrulated normally.

These data show that for a given time dosage, the percentage of embryos poisoned by oxygen varies directly with the partial pressure of that gas. This, of course, is consistent with the evidence presented in a previous section that the role of pressure in effective oxygen treatment lies in its increasing the oxygen concentration in the egg medium.

c. *Pressure-time-dosage relationships*

Increased duration of treatment compensates for reduced partial pressure of oxygen. The most extreme demonstration was the experiment in which a dosage of 1.2 atmospheres resulted in gastrular blockage with 88 hours of treatment. Thus, for a given effectiveness of oxygen poisoning in terms of per cent normal gastrulae, pressure dosage varies inversely with time dosage. This may be seen by extending a horizontal line through the 2 clusters of Figure 8 and comparing their time and pressure dosages at that level.

3. Rate of Development

In almost every experimental run, the stages of the embryos were determined at one or two developmental ages before gastrulation. Each run provided one or more sets of one untreated, or control, and one oxygen-treated sample, each set representing a different parental cross. The members of a given set were of equal sample sizes. At the times of comparative staging, the two samples in each set had the same history of temperature environments. The results of 35 stagings on 22 crosses are collated in Table VI.

In those cases in which retardation did occur, it was of the oxygen-treated eggs. With a few exceptions (cross no. 9, 10, 21), retardation did not occur in those 11 sets in which the treated samples went on to show some percentage of normal gastrulae. Even in the exceptional cases, the retardation appeared only in the later (B) staging. In 11 crosses providing 17 stagings, the oxygen treatment resulted in 0% normal gastrulae. In 7 of these 11 crosses, the treated sample

Table VI

Comparison of developmental stages of oxygen-treated and untreated embryos, the samples of a given cross having the same history of temperature environments at the time of staging. All samples in a given run fertilized at the same time. One treated and one untreated sample per cross. Staging shortly after decompression denoted by letter A; after selection by letter B (cf. Table I). About 300 eggs per sample at A staging; 100 eggs per sample at B staging except for cross no. 18 where 50 eggs were used and cross no. 21 where 192 treated and 200 untreated eggs were used. All samples shaken at 30 c.p.m.

Run no.	Cross no.	Oxygen treatment			Per cent normal gastrulae of treated eggs	Developmental stages of eggs	
		Temperature	Atm.	Hours		Treated	Untreated
1	1B	18.0° C.	3.2	16	0	9−	9
2	2B	"	"	9	43	8+	8+
3	3B	"	"	11	0	9−	9−
4	4B	"	"	"	71	9−	9−
	5B	"	"	"	81	9−	9−
	6B	"	"	"	0	9−	9−
5	7A	"	"	10	0	8+	8+
	7B	"	"	"	"	9	9+
	8A	"	"	10	0	8+	8+
	8B	"	"	"	"	9	9+
	9A	"	"	10	78	8+	8+
	9B	"	"	"	"	9	9+
	10A	"	"	10	10	8+	8+
	10B	"	"	"	"	9	9+
6	11A	"	"	12	0	8	8+
	11B	"	"	"	"	9	9+
7	12A	"	"	11	79	8+	8+
	12B	"	"	"	"	9+	9+
	13A	"	"	11	97	8+	8+
	13B	"	"	"	"	9+	9+
	14A	"	"	11	91	8+	8+
	14B	"	"	"	"	9+	9+
8	15B	12.0° C.	"	14	0	8	8
9	16B	"	"	12¾	0	8+	9−
10	17B	8.0° C.	"	12½	94	8+	8+
11	18A	"	"	23½	0	5	6
	18B	"	"	"	"	7+	8

Table VI (*Continued*)

Run no.	Cross no.	Oxygen treatment			Per cent normal gastrulae of treated eggs	Developmental stages of eggs	
		Temperature	Atm.	Hours		Treated	Untreated
12	19A	8.0° C.	3.2	13¼	0	4	4
	19B	"	"	"	"	9−	9−
13	20A	"	2.2	23	5	7	7
	20B	"	"	"	"	8	8
14	21A	"	"	19	47	6	6
	21B	"	"	"	"	9−	9
15	22A	"	1.2	88	0	9−	9
	22B	"	"	"	"	9−	9+

was retarded. Of the 17 stagings, in 10 cases the controls were ahead of the treated animals.

Where retardation did appear (in 13 of the 35 stagings), it was slight. Except for one staging (no. 18A), the delayed samples were less than one Shumway (1940) stage behind the controls.

In 10 of the 22 crosses development of the oxygen-treated samples was delayed as compared to that of the controls. However, only in cross no. 18 was retardation shown before blastulation. In all cases where the beginning of the lag could be well localized (cross no. 7, 8, 9, 10), it first appeared in the blastula stage.

The substaging (9 −, 9, 9 +) technique was neither very accurate nor precise from run to run. On the other hand, comparing the stages of the two samples at any given time was quite reliable. In other words, in Table VI, comparison of stages in horizontal rows is more dependable than in the vertical ones. Therefore, it is harder to accurately compare sets of samples from many crosses in order to tell when retardation first appears, than it is to tell in how many of all the crosses retardation does occur, and how slight it is. A few experiments without shaking were performed. Here again, in those cases when it occurred, retardation of the oxygen-treated eggs was slight and during blastulation. In the runs using nitrogen (see Table II) instead of oxygen, the gas-treated samples showed no lag in development.

Comparative staging was not as accurately performed at neurulation when the percentages of normal gastrulae were determined as at the pre-gastrular stages recorded in Table VI. Nevertheless, at that time no significant differences in developmental stage between the control, and treated but unharmed eggs were noticed.

It is seen, therefore, that in many but not all cases, oxygen-treated embryos are retarded as compared to untreated controls. Generally, however, the decrease in developmental rate is slight and begins during the late blastula stage.

DISCUSSION

Amphibian embryos subjected at fertilization or during cleavage to any one of a variety of treatments will not gastrulate. This process seems to be a critical one in early development and if not all, certainly larger percentages of experimentally treated eggs die or first appear abnormal at this stage than at any other. Conditions bringing this about include certain hybridizations (Moore, 1941; Brachet, 1944), CN^- treatment (Spiegelman and Moog, 1945), parthogenesis (Parmenter, 1933), and uterine over-ripening (Briggs, 1941). The developmental block produced by oxygen pressure in *R. pipiens* embryos is another which is manifested at or near gastrulation.

The normal development demonstrated in the experiments using nitrogen pressure, some of those using oxygen pressure without shaking, and those of Nelsen (1948) using air pressure, shows that the inhibition of gastrulation through the use of oxygen pressure is not a mechanical effect; oxygen poisoning is not the result of the exertion of a high force per unit area. This is not surprising, for living systems are relatively unaffected by non-localized pressures applied and released gradually (Heilbrunn, 1952, pp. 503–509). With few exceptions, the lowest such pressures having a biological effect are about 100 times those used in the present studies.

At a given temperature the oxygen tension of the embryos' culture medium is directly proportional to the partial pressure of the gas (Dalton's Law) when equilibrium is established, and the results of these experiments can be explained by assuming that the gastrular abnormalities studied are, in turn, functions of oxygen tension. This assumption is confirmed by the experiments using oxygen with and without shaking. Equilibrium between gas and liquid phases is more rapidly established with shaking and the oxygen tension quickly reaches its saturation level in the 10% Ringer's solution. Thus for threshold durations of oxygen treatment, gastrular blockage occurs only in the shaken embryos. As would be expected, non-shaken embryos will be affected only if they are treated with oxygen for longer periods of time. Furthermore, with higher oxygen pressure, the percentage of normal gastrulae falls (see Figure 8). Additional confirmation is provided by experiments of Nelsen (1949) which have been repeated by the present writer. These employed a vertically suspended string-like mass of eggs exposed to oxygen pressure without shaking. Those at the top of the string near the surface of the medium, and therefore in contact with a saturated solution of oxygen, stopped developing at gastrulation. The lower the position of the embryos and, correspondingly, the lower the oxygen tension, the more normal were their fates at gastrulation.

The dosage studies reveal a threshold whose significance is not clear. For, it takes a relatively long duration of treatment (about 8 hours) to produce any abnormal gastrulae; yet after this dosage is completed, treatment of only about 4 more hours results in no normal gastrulae.

Frequently the dosage necessary to produce 100% completely blocked gastrulae is greatly exceeded. Yet developmental arrest never occurs earlier than at the late blastula stage. This suggests that until the very end of the pre-gastrular period, as opposed to the post-gastrular stages, normal development of the embryo is not dependent upon systems which are sensitive to high oxygen tensions.

The developmental rate studies confirm this view. Almost without exception, oxygen-treated embryos develop at the same rate as do untreated ones—until late blastulation. At that time, some, but not all, embryos are slightly retarded. This is understandable if some system sensitive to high oxygen tension, although necessary for normal development after gastrulation, may be dispensed with before this process if, indeed, it operates during the pre-gastrular stages at all.

It should be pointed out that results of this sort are not obtained with all agents causing developmental arrest at gastrulation. Although certain hybrids, as well as oxygen-poisoned embryos, do stop developing abruptly (Moore, 1941), CN^--treated animals are invariably retarded by several stages beginning in early cleavage (Spiegelman and Moog, 1945). Thus in the case of CN^- it cannot be said that what is being affected at the chemical level is correlated in the normal embryo specifically with the events beginning at gastrulation.

The interpretation for the CN^- experiments may be that before gastrulation the poison inhibits a cytochrome oxidase-limiting system which controls developmental rate to an extent such that the latter is merely decreased. At gastrulation, however, the level of inhibition relative to the heightened energy demands (see Barth and Barth, 1954) is such that development ceases entirely. This is a concept of a *quantitative* change at gastrulation.

With oxygen poisoning the interpretation is that a *qualitative* change occurs at or just prior to gastrulation such that a chemical system comes into play whose operation is necessary for development to proceed, but which is not needed for even unretarded pre-gastrular development. What is inhibited during early cleavage is either this system sensitive to high oxygen tension or the conditions necessary for the system's establishment.

These studies were designed as the preliminary steps toward analyses at the cellular and chemical levels. As to the former, the possibility must be entertained that in oxygen-poisoned embryos, gastrular blockage is mediated through chromosomal aberrations. For, increased oxygen tensions enhance x-irradiation effects (Giles and Riley, 1950), and Conger and Fairchild (1952) showed that the chromosome breakage produced by oxygen in *Tradescantia* microspores was identical to that caused by x-rays. Thus it has been suggested (Gerschman, Gilbert, Nye, Dwyer and Fenn, 1954) that high oxygen tensions act similarly to x-rays.

As for the chemical considerations, Brachet, who has long emphasized the role of —SH in development (1950, pp. 170–184), has suggested that the —SH enzymes are inactivated in the oxygen-poisoned embryos (1949). This seems quite probable for Haugaard (1946), using adult mammalian tissue slices and homogenates, demonstrated a close correlation between susceptibility to inactivation by high oxygen pressure and the presence of essential —SH groups in some 20 oxidative and non-oxidative enzymes. Dickens, also in 1946, presented similar results. Non-protein —SH groups are also affected by oxygen, the rate of oxidation being proportional to the oxygen pressure (Barron, 1955). It is generally believed that the inactivation operates through an irreversible oxidation of —SH to —S—S—.

With this body of work as a guide, a metabolic analysis has been started (Malamed, 1954). It was found that oxygen-treated embryos had the same oxygen uptake rate as controls, from shortly after decompression until the late blastula stage. After this stage the controls continued to rise in respiratory rate. At this point, however, corresponding to the time when all the treated embryos were completely blocked, their rate of oxygen consumption levelled off. It then stayed constant until cytolysis set in, about the time the controls developed tailbuds. These results, the same as obtained with a frog hybrid by Barth (1946), indicate that what the oxygen-sensitive system is needed for is the (aerobic) production of energy, which is in turn presumably necessary for the cell movements or, more properly, the mechanical work which constitutes gastrulation.

I wish to express my appreciation for the encouragement and guidance, at both the theoretical and technical levels, of Prof. L. G. Barth. To Prof. O. E. Nelsen I am indebted for the first stimulation of an interest in embryos and oxygen poisoning. Special thanks are due to Prof. J. R. Gregg, Drs. R. McMaster, J. Reiner, and A. Kostellow for their criticisms and suggestions. Mr. A. Pfeiffer was most helpful in the design and construction of the apparatus.

SUMMARY

1. The effect of oxygen poisoning on gastrulation in *Rana pipiens* eggs has been studied using an apparatus consisting of 6 pressure systems continuous with each other or not, in various combinations. The apparatus permitted the embryos to be kept at constant temperature. Shaking and non-shaking samples could be run simultaneously. Oxygen treatment started before the first cleavage and ended during the early cleavage stages.

2. In the mechanical sense, pressure has no effect on gastrulation, for gastrulation is normal in experiments using nitrogen and in others using oxygen without shaking.

3. The role of pressure is via an increase in the oxygen tension of the eggs' medium, according to Dalton's Law. That gastrular blockage is a function of oxygen tension is shown by comparing results with and without shaking for various durations of treatment and by the higher percentage of abnormal gastrulae with higher partial pressure of oxygen.

4. With shaking and 45 p.s.i. of oxygen added to air at 1 atmosphere, durations of treatment of less than 8 hours are without effect on gastrulation. At this threshold, additional treatment of about 4 hours results in no normal gastrulae.

5. Temperature has little if any (net) effect on oxygen poisoning. This is explained on the basis of several temperature effects which are largely compensatory.

6. With 2.2 atmospheres partial pressure of oxygen a longer duration of treatment is required to affect gastrulation than with 3.2 atmospheres. An effect has been obtained using 1.2 atmospheres.

7. Comparison with controls shows that after oxygen treatment the embryos are not always retarded before gastrulation. When there is a developmental delay, it is slight and does not begin before the late blastula stage.

8. These results are interpreted as follows: at gastrulation a qualitative change occurs such that a new chemical system on which development is dependent comes into play. During early cleavage high oxygen concentrations inhibit either this system or conditions necessary for its establishment.

LITERATURE CITED

Barron, E. S. G., 1955. Oxidation of some oxidation-reduction systems by oxygen at high pressures. *Arch. Biochem. Biophys.*, **59**: 502–510.

Barth, L. G., 1946. Studies on the metabolism of development. *J. Exp. Zool.*, **103**: 463–486.

Barth, L. G., and L. J. Barth, 1954. The Energetics of Development. Columbia University Press, New York.

Brachet, J., 1944. Acides nucléiques et morphogénèse, la polyspermie et l'hybridation chez les anoures. *Ann. Soc. Roy. Zool. Belg.*, **75**: 49–74.

Brachet, J., 1949. L'hypothèse des plasmagènes dans le développement et la différenciation *Colloq. Intern. Centre Nat. Recherche Sci.* (*Paris*), **VIII**: 145–162.

Brachet, J., 1950. Chemical Embryology (translated from the French by L. G. Barth). Interscience Publishing Co., New York.

Briggs, R. W., 1941. The development of abnormal growths in *Rana pipiens* following delayed fertilization. *Anat. Record*, **81**: 121–136.

Clayton, J. W., 1950. Oxygen pressure effects on gastrular movements and tail organization in *Rana pipiens*. Master's Thesis, University of Pennsylvania.

Conger, A. D., and L. M. Fairchild, 1952. Breakage of chromosomes by oxygen. *Proc. Nat. Acad. Sci.*, **38**: 289–299.

Dickens, F., 1946. The toxic effects of oxygen on brain metabolism and on tissue enzymes. 2. Tissue enzymes. *Biochem. J.*, **40**: 171–187.

Gerschman, R., D. L. Gilbert, S. W. Nye, P. Dwyer and W. O. Fenn, 1954. Oxygen poisoning and x-irradiation: a mechanism in common. *Science*, **119**: 623–626.

Getman, F. H., and F. Daniels, 1943. Outlines of Physical Chemistry. Seventh edition. John Wiley and Sons, Inc., New York.

Giles, N. H., Jr., and H. P. Riley, 1950. Studies on the mechanism of the oxygen effect on the radiosensitivity of *Tradescantia* chromosomes. *Proc. Nat. Acad. Sci.*, **36**: 337–344.

Haugaard, N., 1946. Oxygen poisoning. XI. The relation between inactivation of enzymes by oxygen and essential sulfhydryl groups. *J. Biol. Chem.*, **164**: 265–270.

Heilbrunn, L. V., 1952. An Outline of General Physiology. Third edition. W. B. Saunders Co., Philadelphia.

Malamed, S., 1954. Influence of oxygen poisoning on development of frog embryos. *Federation Proc.*, **13**: 93–94.

Moore, J. A., 1941. Developmental rate of hybrid frogs. *J. Exp. Zool.*, **86**: 405–422.

Moore, W. J., 1950. Physical Chemistry. Prentice-Hall, Inc., New York.

Nelsen, O. E., 1947. Oxygen and air pressure effects upon the early development of the frog's egg. *Science*, **106**: 295–296.

Nelsen, O. E., 1948. Changes in the form of the blastopore in blocked gastrulae of *Rana pipiens*. *Anat. Record*, **101**: 710.

Nelsen, O. E., 1949. The cumulative effect of oxygen-pressure in the blocking of gastrulation in the embryo of *Rana pipiens*. *Anat. Record*, **105**: 599.

Nelsen, O. E., 1950. Temperature and the oxygen pressure inhibition of the early development of *Rana pipiens*. *Anat. Record*, **108**: 583.

Parmenter, C. L., 1933. Haploid, diploid, triploid, and tetraploid chromosome numbers, and their origin in parthenogenetically developed larvae and frogs of *Rana pipiens* and *R. palustris*. *J. Exp. Zool.*, **66**: 409–454.

Rugh, R., 1948. Experimental Embryology. Revised edition. Burgess Publishing Co., Minneapolis.

Shumway, W., 1940. Stages in the normal development of *Rana pipiens*. I. External form. *Anat. Record*, **78**: 139–148.

Spiegelman, S., and F. Moog, 1945. A comparison of the effects of cyanide and azide on the development of frogs' eggs. *Biol. Bull.*, **89**: 122–130.

Umbreit, W. W., R. H. Burris and J. F. Stauffer, 1949. Manometric Techniques and Tissue Metabolism. Second edition. Burgess Publishing Co., Minneapolis.

THE PRODUCTION OF TWIN EMBRYOS IN DENDRASTER BY MEANS OF MERCAPTOETHANOL (MONOTHIOETHYLENE GLYCOL)[2]

DANIEL MAZIA[1]

Scripps Institution of Oceanography, University of California, La Jolla, California, and Department of Zoology, University of California, Berkeley, California

The problem of individuation in the earliest embryonic development of certain animal groups resolves itself into questions concerning the interaction of blastomeres. Some transaction *between* the blastomeres determines that the first division will produce an individual composed of two cells rather than two individual embryos. Physical contiguity is a factor by definition, for, in those cases where the blastomeres are capable of producing complete embryos, such "twinning" can always be achieved by complete separation of the blastomeres. But complete physical separation is not necessary for functional isolation of the blastomeres; from studies of echinoid eggs we have a variety of experimental conditions under which twin embryos are produced from sister blastomeres in contact with each other (summarized by Schleip, 1929; Harvey, 1940). The experimental problem is to define the means—not necessarily a single one—whereby adjacent cells can mutually influence or restrict each other's behavior. The question is of interest in research on cell division as well as on developmental problems, and probably has much broader implications relative to the behavior of multicellular systems. In the case of echinoid eggs, it has received a good deal of attention, particularly in studies on cell division, and some of the ideas regarding the mechanisms are reviewed in a paper by Dan and Ono (1952).

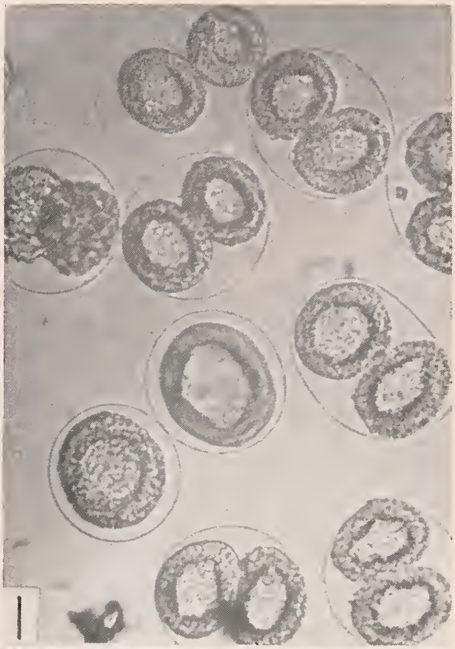
Our chemical insights into the mechanisms of blastomere interaction are rather rudimentary, centering on the study of "extracellular coats" or "intercellular cements" which have, for good reasons, been characterized as calcium proteinates.

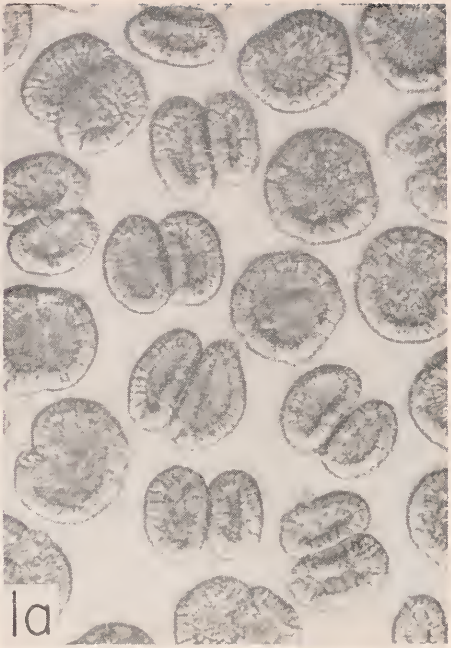
The present work is part of a series of studies in which mercaptoethanol (monothioethylene glycol) was employed as an agent which was expected to interfere with the association of protein molecules through thiol groups. The considerations underlying the study and the selection of this agent are discussed in another paper (Mazia, 1958). It was found, with the eggs of *Dendraster excentricus*, that treatment with mercaptoethanol at the proper time would produce twins in very high yields even though the blastomeres remained in contact within the fertilization

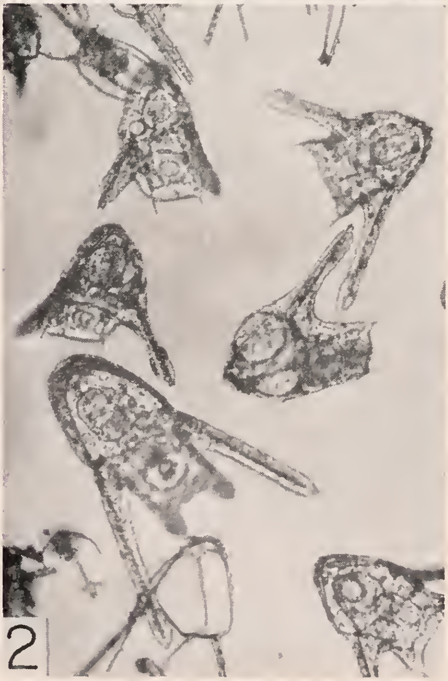
---

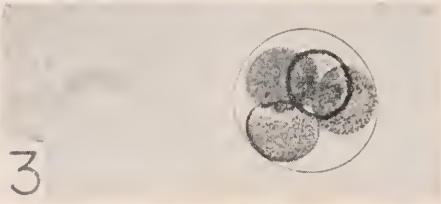
[2] Contributions from the Scripps Institution of Oceanography, New Series, No. 995.

[1] Permanent address: Department of Zoology, University of California, Berkeley. The major part of this work was done during the tenure of a visiting professorship at the Scripps Institution of Oceanography, University of California, La Jolla, California. The author thanks the director and staff of that institution for this opportunity and for their valued cooperation. The work was completed during the tenure of a research professorship in the Adolph C. and Mary Sprague Miller Institute for Basic Research in Science at Berkeley. Material support from the American Cancer Society and the Office of Naval Research is gratefully acknowledged.


1


1a


2


3

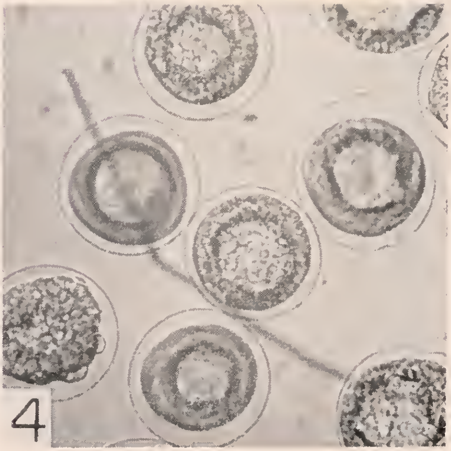

4

FIGURE 1. Twin blastulae. *Dendraster* eggs had been placed in 0.1 *M* mercaptoethanol in sea water at 41 minutes after insemination, and exposed for 28 minutes. Photographed alive at 7 hours 15 minutes after insemination. Twins are hatching in embryo at top of photograph.

membrane. The points of interest in the following discussion are not only the interpretation of the effect as one implicating thiol groups in blastomere interaction, but also the fact that processes determining the twinning or non-twinning may be restricted to a short period *during* the cleavage of the eggs.

METHODS

The details of the methods used will be found in a previous paper (Mazia, 1958). The eggs of *Dendraster excentricus,* obtained in Mission Bay, San Diego, California, were used. At various times after fertilization, nine volumes of egg suspension were mixed with one volume of 1 *M* 2-mercaptoethanol (Eastman) in sea water. A common synonym for mercaptoethanol ($HSCH_2CH_2OH$) is monothioethylene glycol. After various times of exposure, the eggs were washed in sea water and their development was followed. When the fertilization membrane was to be removed, this was done by treatment with a solution of Worthington "Crude Protease" in sea water (0.1 mg. per ml.). In the case of the *Dendraster* egg, the protease may be introduced a few minutes after fertilization, and the dissolution of the fertilization membrane may be observed visually. The fact that the membrane of *Dendraster* eggs is susceptible to protease for some time after fertilization was called to my attention by Dr. William E. Berg.

RESULTS

The over-all study of which this is a part concerned the blockage of mitosis by mercaptoethanol. The essential finding was that 0.1 *M* solutions would block division completely if applied at any time up to the time of metaphase, which takes place at about 35–40 minutes after fertilization, at 24° C. If the mercaptoethanol is applied at any time after this critical point, the cells divide without delay, and if left in the mercaptoethanol are blocked reversibly in the two-cell stage. In the course of observations on the reversibility of the block it was noted that a large proportion of those eggs which had cleaved while in the mercaptoethanol gave rise to twin blastulae when returned to sea water. Such a population containing the twin blastulae is shown in Figure 1. These blastulae gastrulate and develop into normal plutei (Fig. 2).

In order to obtain twinning, the mercaptoethanol must be applied during the period of furrowing. This is shown in Table I, where the yield of twins from eggs placed in mercaptoethanol at various times from metaphase to the completion of furrowing is given. At 35 minutes after fertilization, half of the eggs are blocked

---

FIGURE 1A. Another group of twin blastulae, fixed in 1 per cent formaldehyde in sea water. In this experiment, evidence of incomplete twinning is seen in some individuals.

FIGURE 2. Plutei produced by twinned embryos. The small plutei are the products of twinning. The large pluteus, from an egg which failed to produce twins, serves as a control.

FIGURE 3. Second cleavage of *Dendraster* egg in Ca-free sea water, showing irregular positions of blastomeres. Eggs had been placed in Ca-free sea water at 30 minutes after fertilization and exposed for 60 minutes, during which time the first and second cleavages occurred.

FIGURE 4. Blastulae from eggs that had been exposed to Ca-free sea water from thirtieth to ninetieth minute after fertilization (*cf.* Fig. 3). This experiment paralleled that shown in Figure 1, used the same lot of fertilized eggs and was photographed at the same time as Figure 1. Rotating blastulae gave blurred photographs.

and half have passed into the insensitive stage following metaphase. The latter are blocked in the two-cell stage. Upon return to sea water after 30 minutes in mercaptoethanol, those that had divided in the mercaptoethanol gave rise to twin embryos. Those that were blocked before the first division gave single embryos. By the thirty-eighth minute after fertilization, all of the cells had passed the critical stage, divided in mercaptoethanol and gave rise to a large proportion of twin embryos. Around 45 minutes after fertilization, when most of the cells were well advanced in cleavage at the time the mercaptoethanol was introduced, the yield of twin embryos began to decrease rapidly. If mercaptoethanol was introduced 10 minutes later, the number of twins produced was small.

The critical time for twinning thus comes immediately after the critical time for mitotic blockage, as determined in the previous study (Mazia, 1958). The maximum yield of twins is obtained when the mercaptoethanol is introduced just at the time of the mitotic elongation of the cleavage furrows. The duration of the exposure to mercaptoethanol seems to have relatively little significance. What is important is that it acts during the brief effective period: the 5–10 minutes during which mitosis is completed and furrowing is going on.

TABLE I

Production of twin embryos from Dendraster eggs placed into 0.1 M mercaptoethanol at various times after fertilization

Time after fertilization when mercaptoethanol was introduced* (minutes)	Per cent cleavage in mercaptoethanol	Per cent twin blastulae**
35	50	50
38	90	85
41	95+	90
44	95+	45
47	95+	30
50	95+	8
53	95+	8

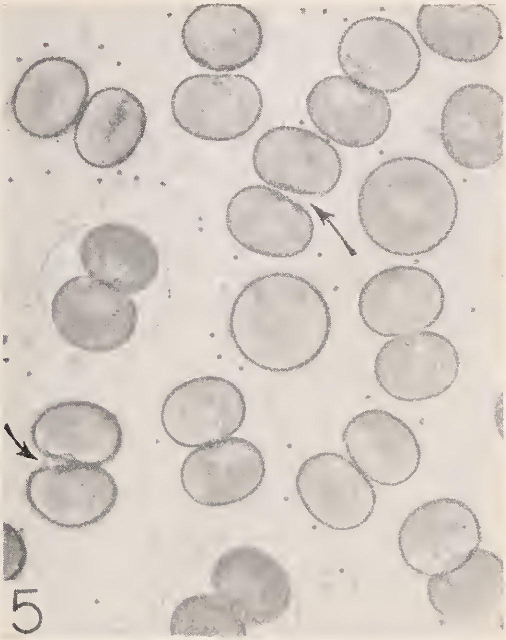
* Duration of mercaptoethanol treatment: 30 minutes.

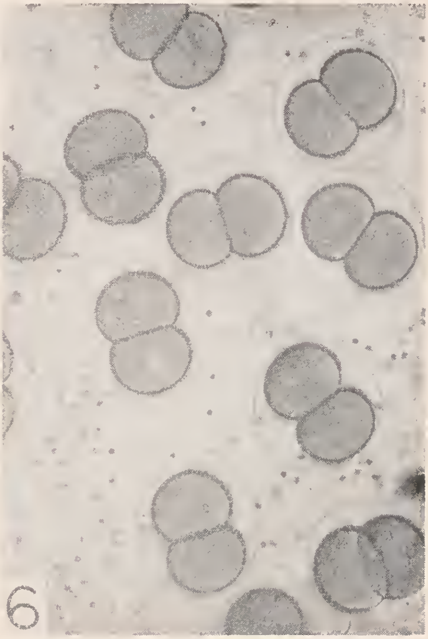
** These percentages are relative to the total number of blastulae, and do not take into consideration individuals which degenerated before reaching the blastula stage. In the particular experiment from which Table I is taken, about 15 per cent of the eggs treated at 38, 41 and 44 minutes degenerated.

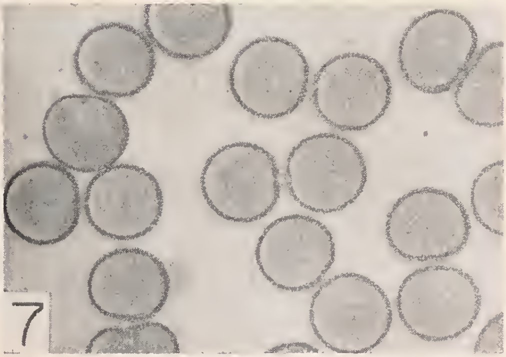
Another kind of variation of "sensitivity" to mercaptoethanol with time should be mentioned, though it has not yet received adequate study. This is evidenced by the failure of some of the eggs to form normal blastulae, single or double. This was not recorded in Table I, where the fraction of twin blastulae is given relative to the total number of blastulae. To illustrate, in the experiment given in Table I, a yield of 90 per cent twins from eggs exposed at 41 minutes after fertilization was given. In the whole population, 15 per cent of the eggs failed to form normal blastulae, so that the yield of twins could also be given as 78 per cent—still a very high figure.

The best-studied methods of obtaining multiple embryos from echinoderm eggs involve modification of the ionic content of the environment, whether by removing

Ca or other ions or by varying the concentration of the sea water in the direction of hypotonicity or hypertonicity. In the present study, the effects of Ca-free sea water were compared with those of mercaptoethanol. A small volume of fertilized eggs (less than 0.5 ml.) was washed by centrifuging and re-suspending in 15 ml. of Ca-free sea water four times, beginning at 30 minutes after fertilization. The







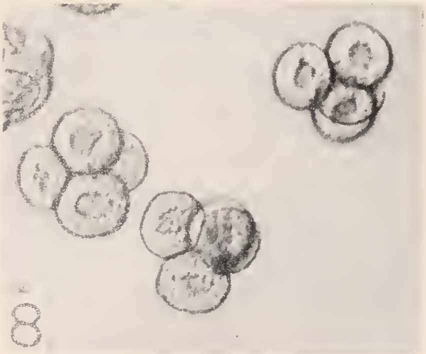


FIGURE 5. *Dendraster* eggs with fertilization membranes cleaving in 0.1 *M* mercaptoethanol. Blastomeres are not so firmly apposed as in control (Fig. 6), but appear to be connected by strands of clear material (arrows).

FIGURE 6. Control for Figure 5. Eggs have just completed cleavage in normal sea water.

FIGURE 7. *Dendraster* eggs without fertilization membranes just after cleavage in 0.1 *M* mercaptoethanol.

FIGURE 8. Quadruplets produced when mercaptoethanol treatment is applied at both the first and second divisions.

fertilization membranes were not removed. The eggs were permitted to go to the four-cell stage in Ca-free sea water before being returned to normal sea water. It was clear by this time that the Ca-deficiency was having its expected effect on blastomere adhesion. The first cleavage blastomeres were not flatly apposed, as was the case in the control, and the planes of the second cleavages did not coincide (Fig. 3). Nevertheless, long treatment with Ca-free sea water did not cause twinning (Fig. 4). Apparently, the embryo can organize itself to form a single blastula, following the treatment with Ca-free sea water, as long as the blastomeres are held together within the fertilization membrane. This corresponds with Harvey's (1940) experience with hypertonic sea water. It should be mentioned that the results in Figure 1 and Figure 4 were obtained with the same lot of eggs.

The mercaptoethanol does visibly affect the adhesion of the blastomeres. Figure 5 shows eggs that have cleaved in mercaptoethanol, the fertilization membrane being present. While they are compressed together, we do see rather more separation than in the controls (Fig. 6), and also see strands of glassy-appearing material between the blastomere surfaces in the furrow. If the fertilization membranes have been removed by protease, the cleavage in mercaptoethanol gives fully spherical blastomeres (Fig. 7), connected by tenuous strands, an appearance almost identical with that of membrane-free eggs that have cleaved in Ca-free sea water.

It would be predicted that if the mercaptoethanol was applied again at the time of the second cleavage, quadruplets would be produced. This was the case, as shown in Figure 8. The yield of quadruplets was never as high as that of twins. This would be expected from the fact that the eggs were not as synchronous in their second cleavage. In a desynchronized population a good many of the embryos will either be in a stage earlier than metaphase, at which they will merely be blocked, or at a stage later than the sensitive part of cleavage (Table 1), in which case the mercaptoethanol will not be effective.

Finally, it should be mentioned that the effect of mercaptoethanol could not be duplicated with ethanol or with ethylene glycol, the analogs lacking the SH group. The latter may be considered the active center, and other SH compounds might have similar effects. The writer has found none that is comparably nontoxic and therefore usable at such high concentrations.

DISCUSSION

Two questions demand discussion: (1) the chemical interpretation of the effect of mercaptoethanol in inducing twinning, and (2) the relation of the results to the earlier observations on twinning and on blastomere adhesion. The most reasonable interpretation of the chemistry of the observed effect is that the mercaptoethanol is affecting some interaction between the blastomeres that involves the formation of S—S bonds. This reagent is commonly used for reducing S—S bonds in proteins (Olcott, 1942). It has been seen that its analogs lacking the SH group are ineffective in inducing twinning. The results would be in accord with the hypothesis that the blastomere interaction depends on the formation of a gel serving as a cement between the blastomeres, and would fit equally well with a hypothesis calling for the establishment of fibrous connections between the blastomeres. The formation of protein gels by the establishment of intermolecular S—S bonds has been described by Huggins *et al.* (1951). The role of S—S bonds in the formation of protein fibers is well known from studies on the keratins. Mercaptoethanol would be expected to block such intermolecular bonding by preventing the oxidation of the SH or by competing with protein SH.

The fact that the mercaptoethanol is effective only during a short period and is ineffective later would lead to the conclusion that we are dealing with the formation of the inter-blastomere links during the cleavage process itself. The mercaptoethanol is effective in preventing the formation of the links but not in splitting them once they are formed. This might mean that it acts by competition with protein SH in the formation of S—S bonds, not by reduction of S—S. It might also mean that the protein S—S becomes inaccessible to the reagent or that sufficient secondary bonds are formed, following the establishment of the intermolecular S—S links, to hold the structure together in the face of the reduction of the latter. In any event, the results imply that during cleavage, connections are formed between the blastomeres. The alternative explanation is that pre-existing factors tending to hold the blastomeres together (*e.g.* the hyaline layer as envisaged by Dan and Ono, 1952) undergo a change that renders them susceptible to mercaptothanol during the brief period of cleavage.

These results do not stand in contradiction to any of the previous observations regarding the induction of twinning by other means and especially by variations in the ionic environment. These have been interpreted, with some difference of opinion as to the details, as reflecting the significance of extracellular layers having the character of calcium proteinates (Moore, 1949; Hagström and Hagström, 1954). The physical properties of such layers are known to be affected by the ionic composition of the medium; obviously they will also depend on the protein-to-protein links that make their existence as stable masses possible. The contrast between the effects of Ca-free sea water and of mercaptoethanol in the present experiments is explicable on the assumption that the Ca-free sea water softens the layers but does not dissolve them quickly, while the mercaptoethanol actually solubilizes newly-appearing or pre-existing protein that would normally function in holding the blastomeres together. Thus the effect of Ca-free sea water might be reversible where the effect of mercaptoethanol was not.

The most striking feature of the mercaptoethanol effect is its complete irreversibility with respect to the division during which the reagent was applied, and its complete lack of effect on subsequent divisions. If it is applied at first cleavage, the blastomeres are "isolated" but subsequent divisions are normal, and the end result is fully normal twins in a large proportion of the population. If it is applied again at the second division, there is a fair yield of normal quadruplets. The results are not perfect: some incomplete twinning and occasional quadruplets are observed when the treatment takes place at the first division and some triplets are obtained when the treatment is given at the first and second divisions. On the whole, however, the results may be described as the effective and irreversible isolation of blastomeres by chemical means.

SUMMARY

1. When *Dendraster* eggs are permitted to cleave in 0.1 *M* mercaptoethanol in sea water and then restored to normal sea water, a large proportion of the embryos develops as twins, producing normal twin plutei.

2. The effectiveness of the mercaptoethanol treatment is restricted to the short period during which the first cleavage furrows are forming.

3. If the treatment is repeated at the time of the second cleavage, quadruplets are produced.

4. Twins are not produced when the eggs cleave in Ca-free sea water.

5. The results are discussed in terms of the significance of the thiol groups of proteins for the interactions of blastomeres.

LITERATURE CITED

Dan, K., and T. Ono, 1952. Cyto-embryological studies of sea urchins. I. The means of fixation of the mutual positions among the blastomeres of sea urchin larvae. *Biol. Bull.*, **102**: 58–73.

Hagström, B., and Britt Hagström, 1954. The action of trypsin and chymotrypsin on the sea urchin egg. *Exp. Cell Res.*, **6**: 532–534.

Harvey, E. B., 1940. A new method of producing twins, triplets, and quadruplets in *Arbacia punctulata*, and their development. *Biol. Bull.*, **78**: 202–216.

Harvey, E. B., 1956. The American *Arbacia* and Other Sea Urchins. Princeton University Press, Princeton, New Jersey.

Huggins, C., D. F. Tapley and E. V. Jensen, 1951. Sulphydryl-disulphide relationships in the induction of gels in proteins by urea. *Nature*, **167**: 592–593.

Mazia, D., 1958. SH compounds in mitosis. I. The action of mercaptoethanol on the eggs of the sand dollar *Dendraster excentricus*. *Exp. Cell Res.* (in press).

Moore, A. R., 1949. On the precursors of the fertilization and the hyaline membranes in the eggs of the sea urchin *Stronglycentrotus purpuratus*. *Biodynamica*, **6**: 197–212.

Olcott, H. S., 1942. Monothioglycol. *Science*, **96**: 454.

Schleip, W., 1929. Die Determination der Primitiventwicklung. Akademische Verlagsgesellschaft, Leipzig.

INHIBITORS OF REGENERATION IN TUBULARIA[1]

KENYON S. TWEEDELL

Department of Zoology, University of Maine, Orono, Me.,
and Marine Biological Laboratory, Woods Hole, Mass.

When stems of *Tubularia* are removed from the colony and isolated from their hydranths, regeneration will occur in the isolated stems preferentially at the distal ends as regulated by an inherent polarity gradient (Child, 1941). Several factors, both intrinsic and extrinsic to the isolated stem, may govern and in many cases prevent regeneration. Of the parameters known to have an inhibitory effect, lowering the temperature will decrease the rate of regeneration (Moore, 1939; Moog, 1941; Berrill, 1948) but it increases the size of the reconstituting hydranths (Moog, 1941). Similarly, Torrey (1912), Miller (1937, 1939), Barth (1937, 1938, 1940), and Rose and Rose (1941) all found that a lowering of the oxygen tension will inhibit regeneration. However, certain respiratory poisons such as cyanide or urethane (Moog and Spiegelman, 1942) will inhibit regeneration without any parallel effect on respiration. Miller (1939) and Goldin (1942a) indicated that increased hydrogen ion concentration of the sea water would reverse the normal polarity of the stems or inhibit regeneration. Later, Goldin (1942b) found that at oxygen tensions favorable to regeneration, an increase of the hydrogen ion concentration by the addition of CO_2, would cause complete inhibition. It was shown by Rose and Rose (1941) that oxygen alone will not assure regeneration unless there is sufficient cut surface of the stem open to the sea water to allow release of an inhibitor, believed to be produced by tissues of the adult organism. Similar results were reported by Goldin (1942a) using explanted coenosarc fragments and Miller (1942) who varied metabolic exchange by covering portions of the perisarc. This inhibitor, presumably a metabolic substance, was collected by Rose (1940) from colony water and later (Rose and Rose, 1941) produced from an aerated, saturated mixture of cut stems and hydranths in sea water. When this water was applied to freshly amputated stems, regeneration was blocked. The active factor was rather unstable, being heat-labile but non-volatile. Hydranths alone were found to be active but there was evidence that stems also produced a substance which made them inhibitory upon one another. Later, Steinberg (1954) showed by ligaturing stems at intervals after amputation that inhibitor production within the regenerating stem begins around 30 hours post-amputation, when the distal hydranth has become well determined.

Recently, Tardent (1955) has been able to produce inhibition with "hydranth equivalent" extracts made from tissue breis of mature hydranths of *Tubularia larynx*. This substance does not lose its activity after sterilization or refrigerated storage.

While the tissue extracts and the inhibitor water have the same effect, namely general inhibition of regeneration, certain evidence suggested that the two factors

---

[1] This investigation was supported in part by the Coe Research Fund, University of Maine.

were not identical. The present experiments were designed to localize the source and further identify the active factor in inhibitor water, and secondly to compare it with the inhibitory factor produced from tissue extracts of the adult organism.

Materials and Methods

Throughout the experiments, *Tubularia crocea* collected in the Woods Hole area was used. Since its appearance can be greatly altered according to the time of year it is collected and the prevailing seasonal conditions, often considerable difficulty is attached to its identification. As originally described by Agassiz (1862) and later by Nutting (1899) and Fraser (1944), *Tubularia* (*Parypha*) *crocea* Agassiz grows from a dense stolon mass and is separated into long pale, almost white stems from 8 to 10 cm. high. The stems are unbranched or slightly branched, annulated sparsely at intervals and the pedicel is distinctly swollen just below the base of the hydranth. The hydranths are red, with 20 to 24 proximal and the same number of distal tentacles. The gonophores (when mature) hang in long racemes between the proximal tentacles. They consist of 10 to 12 slender branches, each of which by successive branching may bear up to 8 or more medusae. The medusae are sessile, with no apparent radiating canals. At the oral end of the female medusae there are 6 to 10 crested tentacles or apical processes which are laterally compressed. The male medusae do not possess these crested structures. The coelenteron may have one or more longitudinal endodermal partitions which form two to four incomplete channels.

Additional observations have shown that the amount of branching found in this species ranges from thickly branched specimens (often caused by settling actinulae) to almost totally unbranched individuals. The mature hydranth may measure 8 to 15 mm. from tip to tip of the proximal tentacles.

Secondly, as observed by Cohen (1952) and Rose (1957) the pigmentation of the hydranth can vary from red through various intergrades of orange and yellow. In the past three summers we have noted hydranths viewed with incident light ranging from rose to orange red earlier in the summer along with various intergrades of orange, yellow or white later in the summer. The latter material tends to have long pale stems and is very sparsely branched. The color in the proboscides of the medusae usually conforms to the color of the hydranth. Another late summer variety conforming to the above description has deep red-wine colored hydranths which often exhibit medusae with a brown or golden proboscis or "core" in contrast to the hydranth color. One important difference of the late summer varieties is their resistance to higher temperature. When the sea water temperature reaches about 21° C., the former variety dies out and the warm water forms survive.

Collection of inhibitor water

Inhibitor water was obtained from stems amputated from the stolon mass with the hydranths left intact. Each stem was cut separately and transferred to fresh sea water accompanied by a minimum of debris, small organisms, etc. The hydranths were washed in filtered sea water and then transferred to an aspirator flask containing twice-filtered sea water. The number of mature hydranths varied from two to four per ml. of collecting fluid.

Air bubbles which kept the stems turning over continually were generated through the flask in one of two ways. Initially, the top of the aspirator bottle was attached to a faucet vacuum aspirator and the base of the flask fitted with a clamp-regulated tube for the air intake. Later, an ordinary aquarium aerator was attached directly to the base spout of the bottle. In both cases the bottle was inclined with the spout down and submerged in a pan of running sea water. In this manner, during operation of the pump the hydranths and stems were continually rotated and aerated. Inhibitor water was harvested after 18 to 24 hours.

The water collected was then filtered twice through No. 1 and No. 50 Whatman filter paper in a Buchner funnel, before being submitted to any other treatment. This will be referred to as plain filtered inhibitor. This solution appears slightly opaque and has a distinctive pungent odor. Microscopic examination shows that breakdown products of cellular cytolysis, bacteria and ciliate protozoans are present.

Preparation of tissue extracts

Large numbers of mature hydranths (250 to 550) were collected, washed in sterile sea water, drained and homogenized in a teflon-glass tissue homogenizer. The resultant brei was then centrifuged for 15 minutes at 1560 G. Several layers were produced. Floating at the top was a tough, dark red foam layer of intact cells, fibers and pigment, immediately followed by a short cap of fatty material. The major portion consisted of an opaque brown supernatant solution and at the bottom there was a dark red pigment layer covered by a white layer. The supernatant solution was re-centrifuged at 15,000 G for 15 minutes. The first and second sediment layers were re-suspended in filtered sea water and again centrifuged at 15,000 G for 5 minutes. The combined supernatants were re-centrifuged at 21,000 G for 15 minutes. The final supernatant was then made up to 100 ml. in filtered, bacteria-free sea water. In the final solution, each ml. of extract was equal to a known number of hydranths depending on the original number.

Throughout the following experiments, the criterion for regeneration was the degree of differentiation, *i.e.,* the number of fully differentiated hydranths per input of freshly amputated stems in standing sea water.

Stages of regeneration referred to are adopted from those described in detail by Davidson and Berrill (1948), Rose and Rose (1941) and Steinberg (1954). They are referred to as the inactive stage, pigmented band (primordia of the tentacles), proximal ridge (proximal tentacle striations), proximal-distal ridge (proximal-distal tentacle striation), pinched (constriction of the hydranth) and emerged (fully developed regenerate) stages.

RESULTS

Before attempting to analyze the active substance in inhibitor water it was deemed necessary to determine from which tissues the inhibitor originated and which were the best sources. This necessitated finding if there was any mutual inhibitory effect of the cut stems upon each other. Both Rose and Rose (1941) and Barth (1938) pointed out that crowding freshly amputated stems would retard their regeneration.

In this experiment, a series of Stender dishes containing 18 ml. of standing sea water were filled with increasing numbers of freshly amputated stems. The results of 6 experiments can be seen in Figure 1. All of the stems regenerated in the dishes up to 16 stems per volume and at 24 stems per dish, at least 90% of the cut stems went on to form fully differentiated hydranths at the same time as the controls, about 48 hours post-amputation. Beyond this, the number of regenerates slowly dropped off. Above an input of 24 stems, the stems which were able to regenerate, did so at a considerable time after the controls. The last two points are readings taken 70 hours after amputation. While it was remarkable that so many stems would regenerate under such crowded conditions, the rate of regeneration had clearly lagged.

These results suggested that if an inhibitor was being produced, it was occurring in sub-threshold quantities or it must come from more differentiated tissues of the regenerating hydranth. This seemed to validate an earlier report by Steinberg (1954) that only later stages of differentiation produce substances which inhibit earlier stages of regenerating stems.

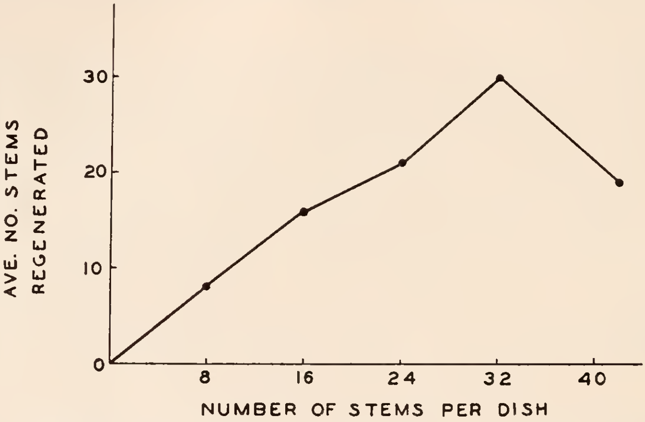


Figure 1. The effect of crowding upon regenerating stems in standing sea water.

Living tissue explants

It had also been reported by Rose and Rose (1941) that the presence of living adult hydranths almost completely inhibited the regeneration of stems. The effect of mature hydranths alone was therefore tested on a relatively simple level of biological assay. Fixed numbers of freshly cut stems were added to standard volumes of standing sea water. To these dishes, freshly amputated hydranths were added in increasing numbers. The purpose was to find the minimum number of hydranths which would show an inhibitory effect upon the regenerating stems. This group of experiments was conducted at temperatures between 18° and 22° C.

In the first series of experiments, amputated hydranths only were placed in 18 ml. of standing sea water in Stender dishes. The number of amputated stems added was either 1, 2, 4 or 8 stems per dish as depicted by solid lines in Figure 2. Each point represents the average of six experiments. There was no inhibitory effect up to the addition of 4 hydranths per dish but as the number of freshly amputated hydranths was increased, the number of regenerates began to decrease.

Between the addition of 16 to 32 hydranths, the maximum number of regenerates was around 3 regardless of stem input. Complete inhibition of all stems occurred with the addition of 40 or more hydranths. This is in agreement with Tardent (1955) who found that tissue extracts of adult hydranths in approximately the same volume of sea water produced almost total inhibition with the addition of 40 hydranth equivalents.

When the volume of the culture medium was increased ten-fold, as represented by the dotted curve B in Figure 2, the first indication of inhibition was seen with the addition of 16 hydranths per bowl with 8 stems. Three out of 8 stems were still able to regenerate along with 64 hydranth explants.

In a second series of experiments the effects of explanted cut stems with intact hydranths upon freshly amputated stems were examined. Total prevention of regeneration became evident when the ratio of tissue explants to regenerating stems became 4:1.

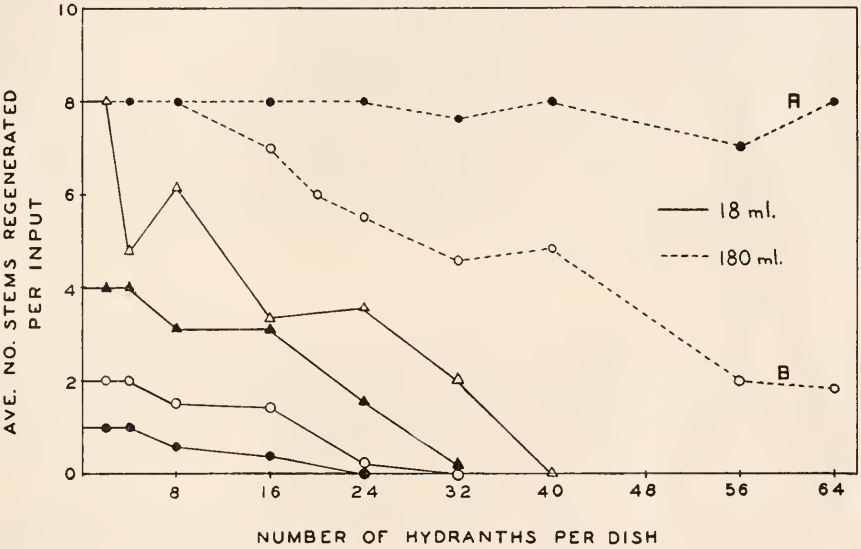


FIGURE 2. The effect of living hydranth explants upon increased numbers of regenerating stems in standing sea water. Each point represents the average of six experiments.

In all cases the stems which were inhibited almost always stopped their development at the stage when proximal and distal ridges were first becoming apparent. The stems which did regenerate in the presence of an increased amount of hydranth material, did so at a considerably later time than the controls. It was obvious that the rate of regeneration was retarded.

Inhibition occurred only when freshly cut stems or those in the very early stages of regeneration were tested. If the previously amputated stems had reached the stage of proximal ridge or later before being added to the culture medium, they were unaffected by the addition of hydranths. The sensitive period to the explants appears early in the regenerative phase.

These results suggested that inhibition might be caused by either an increase in tissue mass which could result in an accumulation of metabolites, a reduction in the oxygen tension or an accumulation of CO_2. It has already been pointed out that a reduction in available oxygen and an increase in CO_2 will prevent regeneration.

Subsequently a duplicate series of dishes were set up in which the culture medium was aerated. Each dish contained 180 ml. of standing sea water and 8 stems. The dishes were aerated with an aquarium aerator and air stones. A comparison of aerated (curve A) and the non-aerated series (curve B) can be seen in Figure 2. Whereas inhibition of regenerates becomes evident in the non-aerated dishes, the effect of additional hydranths, up to the limit studied, was abrogated by aeration in all cases. The principal effect of aeration is to drive off CO_2 from the water (Emmens, 1953) and the above experiments strongly indicate that it is the factor acting here.

Since inhibitor water is usually collected in the presence of vigorous aeration, it did not seem likely that the results above were due to the same factor that is collected in inhibitor water. This conclusion was supported when identical groups of hydranths without stems were placed in standing sea water. After 24 hours, the culture solutions were harvested minus the hydranths and freshly amputated stems were added to the solution. None of the harvested solutions had any apparent inhibitor action upon the regenerating stems. It can be concluded that retardation and prevention of regeneration from both crowded stems and the addition of extra living hydranths to amputated stems are not due to the same factor which is found in inhibitor water.

Effects of inhibitor water

From a practical standpoint, the most effective source of active inhibitor water was from cut stems with the hydranths intact, obtained by the method already described. Repeated harvests of sea water obtained from cut stems only, without the hydranths, were collected under identical conditions. These solutions had no inhibitory effect when applied to freshly amputated stems.

The following sets of experiments were therefore run concurrently from repeated harvests of inhibitor water. The results from each modification of the inhibitor water are treated separately and the tabulations represent single experiments of ten amputated stems each.

Effect of plain filtered inhibitor. In each experiment, 50 ml. of newly collected inhibitor were applied to a series of freshly cut stems, 10 stems per finger bowl. The inhibitor solution used in some experiments completely inactivated most of the stems or arrested development prior to complete regeneration in the remaining stems. Other solutions caused only inactivation of some stems and retarded regeneration in other stems. Partial or total inhibition was in general correlated with the length of collecting time and the number of equivalent hydranths per ml. of collecting fluid. Occasionally a weak inhibitor solution was produced if the number of hydranths/ml. were two or less. Strong inhibitor solutions were always obtained when three or more hydranths/ml. were used.

The results in Table I-A show that when most control stems had completely differentiated or emerged, the majority of the inhibitor-treated stems remained inactive. After 50 to 70 hours post-amputation, many of the stems which had begun a retarded development reached the pinched or emerged stage. Only 9%

of the treated stems actually emerged. It can be seen further that once the treated stems were inactivated, only 7% of them recovered to begin regeneration. It was found that if these inactivated stems are removed and placed in running sea water, they will recover and go on to regenerate.

TABLE I

Comparative effects of treated inhibitor water upon regeneration of cut stems in standing sea water

Type of inhibitor preparation	Hours after amputation	Number in regenerative stages						
		Emerge	Pinch	Prox.-dist. ridge	Prox. ridge	Pigmen. band	Pre-primordia	In-active
A. Plain filtrate	28–46	5	2	1	5	27	28	132
	50–70	18	26	11		22	6	118
Control	30–52	56	41					3
	52–69	79	18					3
B. Dialysis of plain filtrate	30–46	2	2	4				52
	65–70	5	3					52
Control	45	15	3					2
Dialysis control	50	12	6	2				
C. Norite "A" adsorption of plain filtrate	40–70	28	9					2
Filtered inhibitor	40–70	7	8			5		29
Control	40–56	30						
D. Bacterial filtrate of inhibitor	32–46	5	6	6		3		70
	56–70	13	7					65
Control	37–48	69	13					7
E. Dialysis of bacteria-free inhibitor	30–46		4	3		2		81
	56–70	10	11	1		5		63
F. Dialysis of bacteria-free inhibitor in sea water plus chloromycetin	32–46	1	9	7		5		30
	56–70	14	18	3		2		23
								3
Control: Stems in plain sea water	45–56	52	8	4				6

Since Goldin (1942a, 1942b) and Miller (1942) had demonstrated that an increase in hydrogen ion concentration results in a decrease of regeneration, portions of the most potent filtered inhibitor were tested for a decrease in the pH. It was postulated that inhibition might be caused by an accumulation of CO_2 in the inhibitor water or, as both Goldin (1942a) and Miller (1942) suggested, by a lowering of the pH due to the accumulation of acid metabolites. Several checks of the pH of plain sea water and freshly collected inhibitor water showed that there was a maximum drop of 0.57 in pH from that of normal sea water, pH 7.96. Part of the inhibition encountered might be the result of this increase in hydrogen ion tension either from CO_2 accumulation or acid-producing metabolites. The present method of collecting inhibitor water by means of vigorous aeration would tend to drive off any excess CO_2 produced in the medium which would minimize it as a source of inhibition. Goldin's figures show that a drop in initial pH of 1.12, by the addition of HCl, only reduced the number of regenerates to 7 out of 10 as compared to 9 out of 10 in the controls. He did not obtain complete inhibition with CO_2 until he had decreased the initial pH of his solutions from 1.3 to 1.8 pH units depending on the oxygen concentration. It is therefore difficult to assess the effect of increased acidity presumably brought about by acid metabolites in the present experiments.

Dialysis of filtered inhibitor. Routine preliminary tests for proteins made upon the filtered inhibitor were in general negative with the exception of a weak ninhydrin positive result. However, the latter result could be caused by various other contaminates in the inhibitor water.

The inhibitory action of plain filtered inhibitor water was therefore tested on newly cut stems after dialysis. Cellulose dialyzer tubing, 1″ flat, was thoroughly washed with running sea water, both inside and out. (This procedure was found absolutely necessary since dialyzer tubing contains a water-soluble plasticizer that is quite toxic to *Tubularia*. In fact, it was found that the rinsings of dialysis tubing would completely inhibit regeneration.) Each tube, containing 25 or 50 ml. of plain filtered inhibitor, was placed in 150 ml. of standing sea water in a finger bowl. In some cases the solution outside of the dialysis bag was a bacterial filtrate of plain sea water. Ten amputated stems were added to each bowl on the outside of the dialyzer tubing. Control dishes of amputated stems in plain bacteria-free sea water and a second control of sea water dialyzed against standing sea water containing stems were included.

The results showed that the inhibitor filtrate does dialyze and the effect of inhibition is still strongly evident after dialysis as indicated in Table I-B. When these results are compared to the inhibition produced after the direct application of the filtrate, it is seen that there was little decrease in its effectiveness even after dilution against 3 to 6 times its volume.

Treatment with Norite. Concurrent with the previous experiments, part of the filtered inhibitor was treated with Norite "A." The mixture was then filtered through Whatman No. 1 filter paper producing a clear filtrate. This solution was then applied to freshly amputated stems. The results are shown in Table I-C. It is clear that adsorption by Norite almost completely removed the effect of the inhibitor. While this treatment did eliminate the factor or factors which cause inhibition, their identity was far from established. Likewise, microscopical examination of the clear filtrate also indicated that cells in suspension and microorganisms were removed.

It was quite possible that the presence of cells or microorganisms was linked to activity of the inhibitor and experiments were devised to eliminate them. Earlier, the plain filtered inhibitor was subjected to high speed centrifugation at 21,000 G for one hour and the supernatant decanted off. Most of the bacteria would be eliminated by this procedure. Application of this solution of cut stems showed no alteration in inhibitor activity. Since Rose (1940) had shown that heating the inhibitor to 90 or 100° C. completely inactivated it, bacterial filtration was utilized.

Action of inhibitor after bacterial filtration. A clear bacteria- and cell-free solution was obtained after passing the inhibitor solution through a Mandler bacterial filter. In each experiment, ten newly amputated stems were introduced into 50 ml. of this solution. While bacteria and other microorganisms are introduced along with the stems, their growth was never observed in the straight filtrate and the solution remained clear.

After 48 hours most of the controls had regenerated but the majority of the stems in the bacteria-free inhibitor were inactive or retarded in their development. See Table I-D. It was observed that many of the stems which began cell movement formed curious bulbular outgrowths or blebs at the distal and sometimes proximal ends of the stem. These abortive attempts to regenerate are evidence that tissue migration does occur but even the early signs of hydranth differentiation are lacking. Even so, a considerable number of retarded stems reached the fully emerged regenerative stage. It was becoming apparent that the active factor in the inhibitor was not dependent upon a continuous interaction with microorganisms. This conclusion was further supported by the next experiment.

Dialysis of bacteria-free inhibitor. In experiments which were run concurrently with those using bacteria-free inhibitor, 50 ml. of bacteria-free preparations of inhibitor were sealed in well washed dialyzer tubing. The tubing was rinsed in sterile sea water and then placed in 150 ml. of standing bacteria-free sea water in finger bowls. The inhibitive action of the bacteria-free inhibitor was still effective after dialysis as shown in Table I-E. When almost all controls had regenerated (from 45 to 56 hours), none of the treated stems had emerged. Here, again, a certain number of the retarded stems were able to recover and regenerated tardily. It can be seen by comparison with the action of filtered inhibitor alone that little activity was lost from the dilution of the inhibitor after dialysis. Since microorganisms were presumably blocked from the bacteria-free inhibitor water contained in the dialysis tubing, there does not appear to be a direct interaction between them and the inhibitor factor.

As a precaution against possible growth of microorganisms introduced on the amputated stems, part of the bacteria-free sea water was prepared with 0.002% of chloromycetin. The results shown indicated that the antibiotic offers some protection against the inhibitor. Whereas only 23% of the stems ever regenerated when treated with the bacteria-free filtrate, 53% of the stems treated with chloromycetin eventually regenerated. The greatest recovery was seen when there was partial inhibition caused by a less active inhibitor. The possible explanation for this unusual result will be discussed later.

Inhibition with adult tissue extracts

Extracts prepared from tissue breis of the entire adult hydranth were made according to the procedure stated earlier and adopted from the technique of Tardent (1955). The hydranth extracts were found highly resistant to heat of sterilization or boiling. Such treatment caused a denaturation of proteins while the remaining filtrate was still active. This filtrate could be refrigerated for several days at 7° C. with no diminution of activity. All subsequent extracts were therefore subjected to heat sterilization and centrifuged to throw down the precipitate. Further high speed centrifugation of the extract at 21,000 G for one hour had no effect on the inhibitor activity.

Tardent's experiments were based on the addition of hydranth equivalents per 15 ml. of culture medium in which he measured the regeneration rate (length/time) of the cut stem. He produced complete inhibition after adding 20 to 40 hydranth equivalents. In experiments designed to duplicate Tardent's, we found that even the addition of one ml. of extract (equivalent to 5 hydranths) would completely stop all regeneration. This amount is much lower than that which Tardent reported necessary for complete inhibition.

TABLE II

Effects of increasing amounts of hydranth tissue extracts on regenerating stems in standing sea water

Hours after amputation	Amount of added sterile extract. 1 ml. = 5 hydranth equiv.	Stages reached					
		Emerge	Pinch	Proximal-distal	Proximal	Pigment band	Inactive
+50	5 ml.				2	4	14 (bulbous)
	7				8		12 (bulbous)
	10						20 (bulbous)
	control	20					
+70	1 ml.	20					
	2	20					
	3	20 (stunted)					
	4	14	6				
	5		2		6	12	
	7	2	4			12	
	10						20
	control	20					
+96	1 ml.	20					
	2	20					
	3	20 (stunted)					
	4	20 "					
	5	20 "					
	7	8	2			10	
	10						20
	control	20					

In a second series of experiments shown in Table II, each bowl contained 10 stems in sea water made up to 100 ml. with increasing concentrations of extract. Each ml. of extract was equivalent to 5 hydranths. It was found that up to the addition of 3 ml. of extract, all stems could regenerate after 72 hours although they were stunted in size. The effective concentration which blocked part of the stems fell between 20 and 25 hydranth equivalents. As the concentration was increased from 5 to 7 ml. ($\frac{1}{5}$ to $\frac{1}{3}$ hydranth equivalent per ml. of culture solution) none of the treated stems had emerged after 50 hours when all the control stems had regenerated. In spite of this, examination of the experimental stems after 96 hours showed that some of the retarded stems were capable of regeneration. Those which did regenerate did so at an extremely slow rate and always resulted in stunted individuals. In particular, the proximal and distal tentacles were smaller. Complete inhibition, with no individuals regenerating, occurred between a hydranth equivalent concentration of 35 to 50 per 100 ml. of culture fluid (a concentration of $\frac{1}{3}$ to $\frac{1}{2}$ hydranth per ml. of the culture medium).

Most of the retarded stems, particularly those in hydranth equivalent concentrations of 25 or higher, manifested large bulbous protrusions, often accompanied by concentrations of pigment at the tip but without other signs of differentiation. These were not unlike those produced by the action of inhibitor water.

Dialysis of tissue extracts. Preparations of full strength hydranth extract (25 ml.) were placed in dialysis tubing and dialyzed against 125 ml. of standing sea water along with 10 freshly amputated stems. Identical preparations of sterilized, precipitated and centrifuged tissue extracts were also tested. From Table III it is evident that both preparations of the hydranth extract can dialyze and inhibit regeneration of the cut stems. Dialysis tubing filled with plain sea water had no effect on the stems. Likewise, if the extract was first dialyzed against running sea water for 24 hours, the subsequent application of the dialysate to regenerating stems showed a total loss of inhibitory activity.

TABLE III

Action of hydranth tissue extracts on regenerating stems after dialysis in standing sea water

Tissue extract preparation	Hours after amputation	Number regenerated
Control (stems only)	+ 50	10/10
Dialysis of plain extract	+ 36	0/10
	+ 50	0/10
Dialysis of boiled, precipitated, and centrifuged extract	+ 36	0/20
	+ 50	0/20
	+ 62	1/20
	+ 68	1/20
	+100	1/20
Dialysis of extract *after* 24 hour dialysis in running sea water	+ 50	7/10
Control—dialysis of plain sea water	+ 37	14/20
	+ 62	20/20

Preliminary identification of the inhibitor. Bacterial filtrates of inhibitor water were prepared and these submitted to general biochemical tests. Routine tests of the filtered inhibitor for protein were negative and it has been shown that the active factor in the inhibitor water is dialyzable. The filtrate consistently gave a positive test with Schiff's reagent, usually regarded as specific for aldehydes. Numerous investigators have questioned this specificity and have suggested that the reagent will react with ketones and other substances with unsaturated hydrogen bonds but the evidence for this is quite conflicting (Hale, 1957). Other tests of the inhibitor solution for ketones were found to be negative.

Since the active factor in inhibitor water could be adsorbed on activated charcoal, preliminary investigations were made with a variety of adsorbants in a chromatographic column. A Pyrex column was employed, 24″ long × $\frac{1}{2}$″ internal diameter, and fitted to a suction flask attached to a faucet vacuum. Occasionally, filtration was aided by a positive pressure head supplied from an aerator.

The inhibitor solutions were first adsorbed on Norite "A" and amberlite resins CG-45 and CG-50 and then eluted with either 2% ammonia in 50% ethanol or 1% acetic acid in sea water. The resultant elutants gave positive tests with Schiff's reagent. Application of the neutralized elutants to freshly amputated stems resulted in inhibition but these results were not conclusive due to the nature of the solvents.

As it was desired to apply these elutants to cut stems for assay, water-soluble adsorbents, magnesium oxide and aluminum oxide were used. When these columns were eluted with sea water, the elutants gave negative tests with Schiff's reagent and still retained some of the inhibitor activity. At this point it is not certain if the test substances positive to Schiff's reagent are identical with the inhibitor fraction. Further chromatographic analyses are anticipated.

Discussion

Throughout the present experiments three principal observations were associated with the inhibition of amputated stems. When the inhibitory effect fell short of causing complete inhibition, a reduction in the rate of regeneration was always noted. This was measured by the length of time necessary for the regenerate to reach the fully differentiated stage of hydranth formation. Such an effect has been reported with almost every inhibitory parameter of regeneration investigated.

Very often reduced rate was accompanied by a reduction in the size of the regenerating hydranths. Generally, size reduction may be correlated with a reduced rate but, as Moog (1941) has observed, a lowering of the temperature allows an increase in the size of the regenerate in *Tubularia*. Any regeneration rate based on length per time could therefore be subject to this and other errors. For this reason the criterion of stages in differentiation was used.

Another characteristic of inhibition was the evident prevention of differentiation even though the stems displayed activity usually associated with it. In many of the non-regenerating stems knob-like blebs of tissue were formed beyond the perisarc at the distal end. Quite often both ends of the stem were so affected. These projections were probably caused by a migration of the coenosarc since the coenosarc became visibly thinner in the center of the stem but they were never accompanied by visible differentiation. A shifting movement of the entire coenosarc toward the distal end in normal regeneration, as reflected in a gradient of optical density, has been thoroughly described by Steinberg (1954, 1955). It is likely that the cellular movement seen here is of the same nature but the prevention of regeneration in the present experiments seems to be a suppression of differentiation rather than a restriction of cell movement.

Inhibition of differentiation in an active regenerate can be produced in other ways. Specific inhibition of differentiated parts has been strikingly demonstrated with living tissue grafts by Rose (1955, 1957). He found if grafts of developing hydranth primordia were properly orientated in a distal position to a regenerating hydranth, the grafts suppressed the differentiation of the specific like parts in the host. The influence of the graft was so strong that it could cause the regression of specific like parts already formed.

A group of experiments have been performed recently by C. Fulton (personal communication) in which he collected inhibitor water in low concentrations of streptomycin or penicillin. In most cases the growth of microorganisms was restricted and the water collected was not effective against regenerating stems. These results suggested that the inhibitor production was linked to microorganisms which are known to multiply during the collection of inhibitor water. In the present experiments precautions were taken against inclusion of microorganisms in the active portion of the inhibitor water but this does not rule out the significance of Fulton's observations, that the activity of inhibitor water might be due to byproducts of bacteria.

However, there are several reasons why the inhibitor effect may not be a direct toxic agent of bacteria or other microorganisms. First, the experiments of Rose (1940), Tardent (1955) and our own show that stems alone, devoid of hydranths, do not produce inhibitor water when collected under identical conditions. Since microorganisms do grow in stem water, this water should also be inhibitory if the bacteria are producing a toxic factor. Secondly, from the present experiments it was seen that inhibition is not always a total all-or-none effect. In a weak solution of inhibitor all stems may eventually regenerate long after the controls. Occasionally 1 out of 10 treated stems will regenerate along with total regeneration in the controls. If the inhibitor were a toxic factor produced by bacteria, it would completely stop all stems from regenerating.

Other observations of Rose and Rose (1941), Steinberg (1954) and our own indicated that the inhibitor acts only during the early stages of regeneration. It is not likely that a toxic substance produced by bacteria would be so specific as to affect only one portion of the regenerative phase.

Lastly, the introduction of antibiotics to the collecting water, in addition to having a bacteriostatic action, might also greatly reduce the effectiveness of the inhibitor. As seen in the present experiments, the addition of an antibiotic, chloromycetin, somewhat suppressed the activity of the inhibitor. Antibiotics are sometimes used to remove biologically active substances from solution. Kutsky (1953, Kutsky *et al.,* 1956), working on the isolation of nucleoproteins from chick embryo extracts, used streptomycin to cause a specific precipitation of the nucleoprotein fraction from the supernatant fluid. The possibility that the same kind of action is occurring when inhibitor water is collected in the presence of antibiotics should not be overlooked.

Summary

1. A study has been made of the effects of living tissue explants, inhibitor water solutions and tissue extracts as inhibitors of regeneration in *Tubularia crocea.*

2. Stems alone have little inhibitory effect upon one another.

3. Living hydranth explants can cause complete inhibition of cut stems. The effective sensitive period or the period of developmental arrest extends from the

time of amputation to just before the proximal ridge stage. Inhibition in these cases can be cancelled by aeration and is not due to metabolic inhibitors.

4. Complete inhibition of cut stems can be produced with harvests of culture solutions taken from cut stems with intact hydranths. No inhibition was obtained with solutions taken from stems only. There is little loss in potency after filtration, centrifugation or sterilization. The active factor withstands bacterial filtration and is dialyzable. The fresh filtrate gives a positive reaction with the Schiff reagent. It is heat-labile, susceptible to cold storage and can be adsorbed on Norite "A." Part of its activity can be removed with antibiotics and it is possible to completely adsorb the inhibitor on inorganic salts and ion exchange resins.

5. The supernatants obtained from breis of hydranth tissue were also an effective inhibitor. A threshold concentration of 20 to 25 hydranth equivalents/100 ml. initiated inhibition and complete inhibition resulted from the addition of $\frac{1}{3}$ to $\frac{1}{2}$ hydranth equivalent/ml. of culture medium. Fresh or boiled tissue extract will dialyze and cause complete inhibition and prior dialysis of the extract against running sea water does alter its potency as an inhibitor. The extract is highly resistant to sterilization, centrifugation and storage.

LITERATURE CITED

Agassiz, L., 1862. Contributions to the natural history of the United States of America, IV, pp. 249–265, 342.

Barth, L. G., 1937. Oxygen as a controlling factor in the regeneration of *Tubularia*. *Biol. Bull.*, **73**: 381.

Barth, L. G., 1938. Oxygen as controlling factor in the regeneration of *Tubularia*. *Physiol. Zoöl.*, **11**: 179–186.

Barth, L. G., 1940. The role of oxygen in regeneration in *Tubularia*. *Biol. Bull.*, **79**: 360.

Berrill, N. J., 1948. Temperature and size in the reorganization of *Tubularia*. *J. Exp. Zool.*, **107**: 455.

Child, C. M., 1941. Patterns and Problems of Development. Univ. of Chicago Press, Chicago.

Cohen, A. I., 1952. Studies on the pigmentation changes during reconstitution in *Tubularia*. *Biol. Bull.*, **102**: 91–99.

Davidson, M. E., and N. J. Berrill, 1948. Regeneration of primordia and developing hydranths of *Tubularia*. *J. Exp. Zool.*, **107**: 465–477.

Emmens, C. W., 1953. Keeping and Breeding Aquarium Fishes. Academic Press, New York.

Fraser, C. McLean, 1944. Hydroids of the Atlantic Coast of North America. Univ. of Toronto Press, Toronto. Pp. 94–102.

Goldin, A., 1942a. Factors influencing regeneration and polarity determination in *Tubularia crocea*. *Biol. Bull.*, **82**: 243–254.

Goldin, A., 1942b. A quantitative study of the interrelationship of oxygen and hydrogen ion concentration in influencing *Tubularia* regeneration. *Biol. Bull.*, **82**: 340–346.

Hale, A. J., 1957. The histochemistry of polysaccharides. *In:* International Review of Cytology, **VI**: pp. 193–263.

Kutsky, R. J., 1953. Stimulating effect of nucleoprotein fraction of chick embryo extract on homologous heart fibroblasts. *Proc. Soc. Exp. Biol. Med.*, **83**: 390–395.

Kutsky, R. J., R. Trautman, M. Lieberman and R. M. Cailleau, 1956. Nucleoprotein fractions from various embryonic tissues; a comparison of physiochemical characteristics and biological activity in tissue culture. *Exp. Cell Res.*, **10**: 48–54.

Miller, J. A., 1937. Some effects of oxygen on polarity in *Tubularia crocea*. *Biol. Bull.*, **73**: 369.

Miller, J. A., 1939. Experiments on polarity determination in *Tubularia* regenerates. *Anat. Rec.*, **75**:(4), Suppl. 38–39.

Miller, J. A., 1942. Some effects of covering the perisarc upon *Tubularia* regeneration. *Biol. Bull.*, **83**: 416–427.

Moog, F., 1941. The influence of temperature on reconstitution in *Tubularia*. *Biol. Bull.*, **81**: 300–301.

Moog, F., and S. Speigelman, 1942. Effects of some respiratory inhibitors on respiration and reconstitution in *Tubularia*. *Proc. Soc. Exp. Biol. Med. N. Y.*, **49**: 392.

Moore, J. A., 1939. The role of temperature in hydranth formation in *Tubularia*. *Biol. Bull.*, **76**: 104–107.

Nutting, C. C., 1899. The hydroids of the Woods Hole region. *Bull. U. S. Fish Comm.*, **19**: 325–386.

Rose, S. M., 1940. A regeneration-inhibiting substance released by *Tubularia* tissue. *Biol. Bull.*, **79**: 359–360.

Rose, S. M., and F. C. Rose, 1941. The role of a cut surface in *Tubularia* regeneration. *Physiol. Zoöl.*, **14**: 328–343.

Rose, S. M., 1955. Specific inhibition during differentiation. *Ann. N. Y. Acad. Sci.*, **60**: 1136–1159.

Rose, S. M., 1957. Polarized inhibitory effects during regeneration in *Tubularia*. *J. Morph.*, **100**: 187–205.

Steinberg, M. S., 1954. Studies on the mechanism of physiological dominance in *Tubularia*. *J. Exp. Zool.*, **127**: 1–26.

Steinberg, M. S., 1955. Cell movement, rate of regeneration, and the axial gradient in *Tubularia*. *Biol. Bull.*, **108**: 219–234.

Tardent, P., 1955. Zum Nachweis eines regenerationshemmenden Stoffes im Hydranth von *Tubularia*. *Rev. Suisse de Zool.*, **62**: 289–294.

Tardent, P., 1956. Pfropf-Experimente zur Untersuchung des regenerationshemmenden Stoffes von *Tubularia*. *Rev. Suisse de Zool.*, **63**: 229–236.

Torrey, H. B., 1912. Oxygen and polarity in *Tubularia*. *Univ. of Calif. Publ. Zool.*, **9**: 249.

Vol. 114, No. 3 June, 1958

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE EFFECTS OF 2560 r OF X-RAYS ON SPERMATOGENESIS IN THE MOUSE[1]

JOHN H. D. BRYAN AND JOHN W. GOWEN

Department of Genetics, Iowa State College, Ames, Iowa

The effects of irradiation on the mammalian testis have been the subject of numerous investigations (for example Regaud and Blanc, 1906; Hertwig, 1938; Eschenbrenner and Miller, 1950; Oakberg, 1955; and Bryan and Gowen, 1956). The more recent papers of this series have been concerned with quantitative aspects of the problem. The foregoing studies have established the fact that the spermatogonia are the most radiation-sensitive constituents of the seminiferous tubules. After exposure to radiation, spermatogonial proliferation is progressively reduced and the frequency of spermatogonia declines to a very low level. Following this irradiation-induced decrease in spermatogonia, the other cell types (spermatocytes, spermatids and sperm) disappear in the order of their development. Knowledge of the nature of the spermatogonial response is therefore of considerable importance to an approach toward an understanding of the action of irradiation on cells and tissues. Evidence has accrued which suggests that spermatogonial necrosis may be an important factor (Regaud and Lacassagne, 1927; Hertwig, 1938; and Oakberg, 1955). In this regard conclusions based on tracer studies must also be considered. The studies of Holmes (1947), Howard and Pelc (1953), Forssberg and Klein (1954), Smellie *et al.* (1955) and others, clearly show that irradiation effectively inhibits DNA synthesis together with mitotic activity. Furthermore the data of Howard and Pelc (1953) indicate that the mitotic inhibition (or delay) is brought about by the failure of cells in interphase to enter the synthetic phase, rather than by interruption of synthetic processes already going on. These studies would suggest that in the case of the testis, the cessation of spermatogonial activity (through inhibition of DNA synthesis) should be a major factor in bringing about the irradiation-induced depletion of spermatogonia. The data of Bryan and Gowen (1956), derived from quantitative histological and from cytophotometric studies, are in accord with these ideas—as are the earlier conclusions of Eschenbrenner and Miller (1950) and Shaver (1953). There are, then, two rather different responses to irradiation which have been advanced as explanations for the observed behavior of irradiated mammalian seminiferous tubules. The important point is: what are the relative levels of importance which may be ascribed to either process? It appeared likely that a comparison of results following exposure to different dose levels of x-rays would shed more light on the nature of any relationship between these proposed mechanisms. In our previous paper results obtained following exposure to 320 r of x-rays were reported. This present paper reports data obtained following exposure to a high dose of x-rays (2560 r). These data are, where feasible, presented together with corresponding data from our 320 r experiment. As will be seen, the available evidence suggests that both mechanisms play a role in the observed radiation response of spermatogonia, the level of importance ascribable to either one depending upon the dosage levels of radiation employed.

---

[1] Journal Paper No. J-3333 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1187. This work has received assistance from Contract No. AT(11-1)107 from the Atomic Energy Commission.

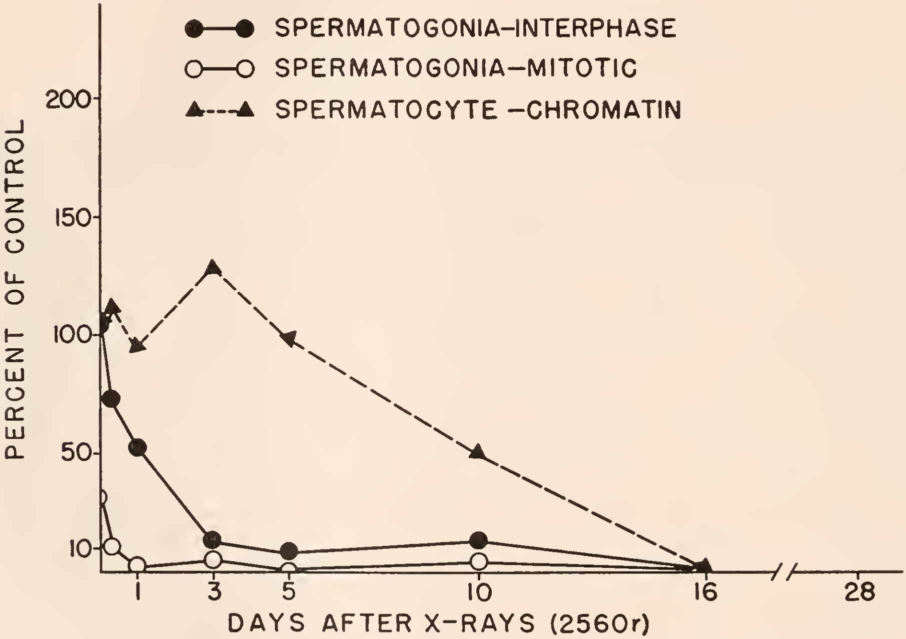


FIGURE 1. Strain Ba. Incidence of spermatogonia and spermatocytes at different times following 2560 r of x-rays.

MATERIALS AND METHODS

The animals chosen were 58-day-old males of strains BALB/Gw (hereinafter referred to as Ba) and S. These inbred strains of mice differ in their sensitivity to mouse typhoid. The experimental animals were irradiated in plastic tubes and were exposed to a dose of 2560 r (250 pkv, 30 ma; filtration 0.25 mm. Cu, 1 mm. Al; anode-target distance 47.5 cm., dose rate 430 r/min.). The irradiation was delivered to the pelvic region only, the rest of the body being shielded with lead. These conditions of irradiation are, except for the x-ray dose, identical with those of our previous studies (Bryan and Gowen, 1956).

Control and irradiated animals were killed at 1, 8 and 24 hours, 3, 5, 10, 16 and 28 days following exposure. The 28-day material is missing from the S series due to death of animals prior to this sampling time. From each animal the testes were rapidly removed and weighed. One testis was then fixed in Carnoy's acetic-alcohol (1:3) and the other used for dry weight determinations.

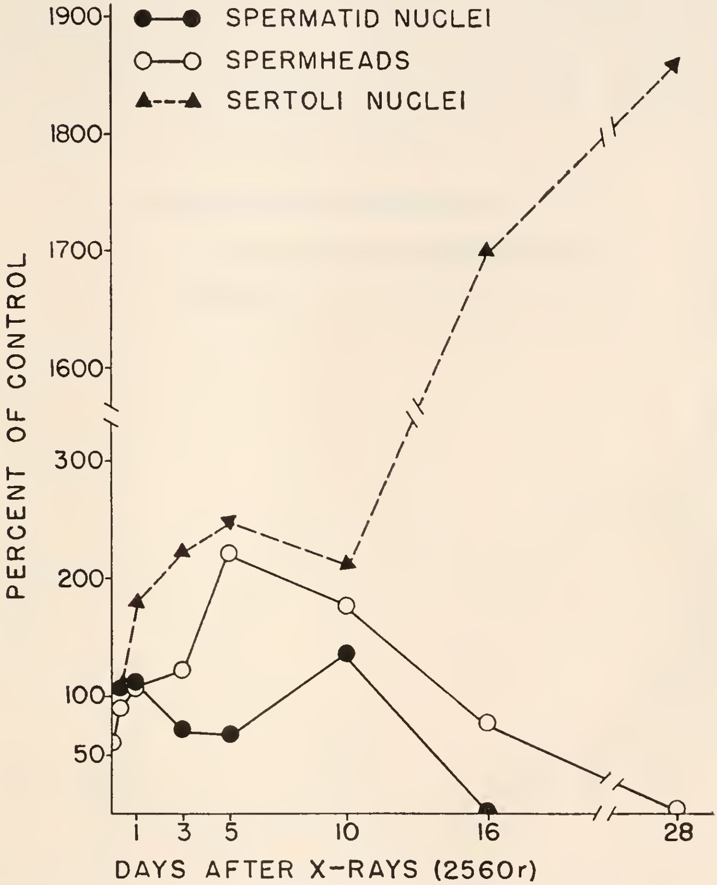


FIGURE 2. Strain Ba. Incidence of spermatids, sperm and Sertoli nuclei at different times following 2560 r of x-rays.

The histological material was processed, and slides were stained, as described earlier (Bryan and Gowen, 1956). As in our previous work, the procedure of Chalkley (1943) was used to obtain estimates of the relative areas of the tubules occupied by each stage of spermatogenesis. This procedure was also used to provide data with respect to spermatogonial and non-spermatogonial necrosis during the first 24 hours following exposure to x-rays.

RESULTS

The data obtained are summarized in Tables I–III. Data pertaining to both strains are presented together for ease of comparison. In Table I all values are expressed in terms of per cent of control values. These data are also expressed in graphical form in Figures 1–4.

The data of Table I indicate that the relative area occupied by spermatogonia in interphase undergoes little change during the first hour after irradiation. Thereafter there is a pronounced and progressive decline which reaches a low point by 5 days following exposure. The data further suggest that there may be an abortive attempt at regeneration during the period of 5–10 days after x-rays, followed by a further decline (absence of spermatogonia at 16 days). With respect to the mitotically active spermatogonia, a marked contrast in response is evident.

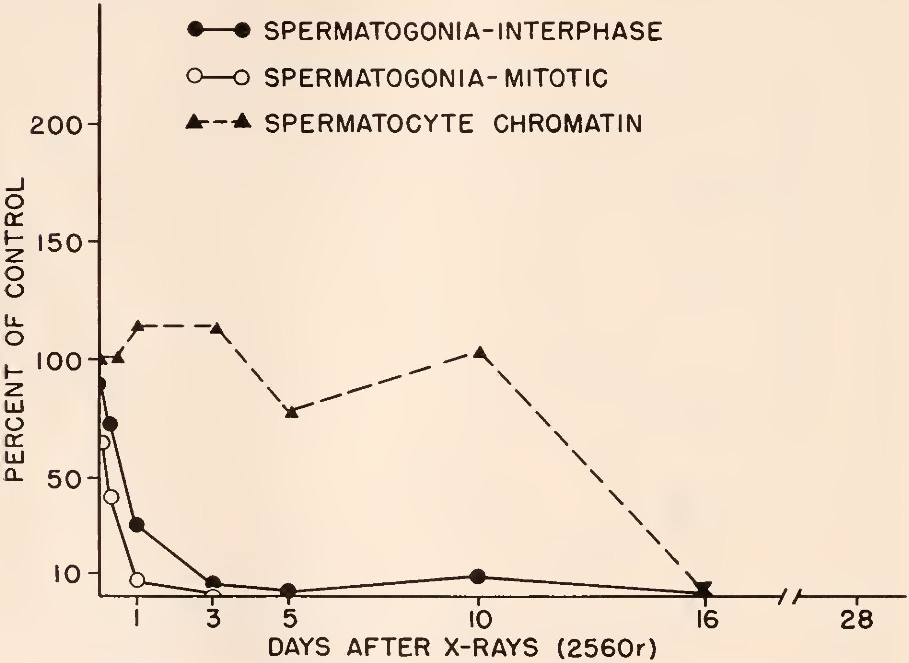


FIGURE 3. Strain S. Incidence of spermatogonia and spermatocytes at different times following 2560 r of x-rays.

Thus by one hour after irradiation the area occupied by this class has declined to 65% of the control in the case of strain S and 30% of the control in strain Ba. In both strains at one day after exposure, the area has further declined to less than 10% of the control value. Thereafter no spermatogonial mitotic activity was recorded for strain S during the remainder of the experiment. In the case of strain Ba, the response is essentially the same except that a low level of spermatogonial activity (4% of control) was encountered in the 10-day material.

Two other cell classes disappear from the seminiferous epithelium following exposure to 2560 r of x-rays. These are the spermatocytes and spermatids. The respective areas occupied by these cells have declined to zero levels by 16 days after exposure.

In view of the recorded differences in behavior of the sperm fraction of the two strains (see Table I, and Figures 2 and 4), it is unfortunate that 28-day material from strain S was not available. The response over the period of 1–10 days is rather similar. Over the period 10–28 days, strain Ba data indicate a progressive decline to a very low level at 28 days whereas strain S, in contrast, shows a marked increase during the 10–16-day period.

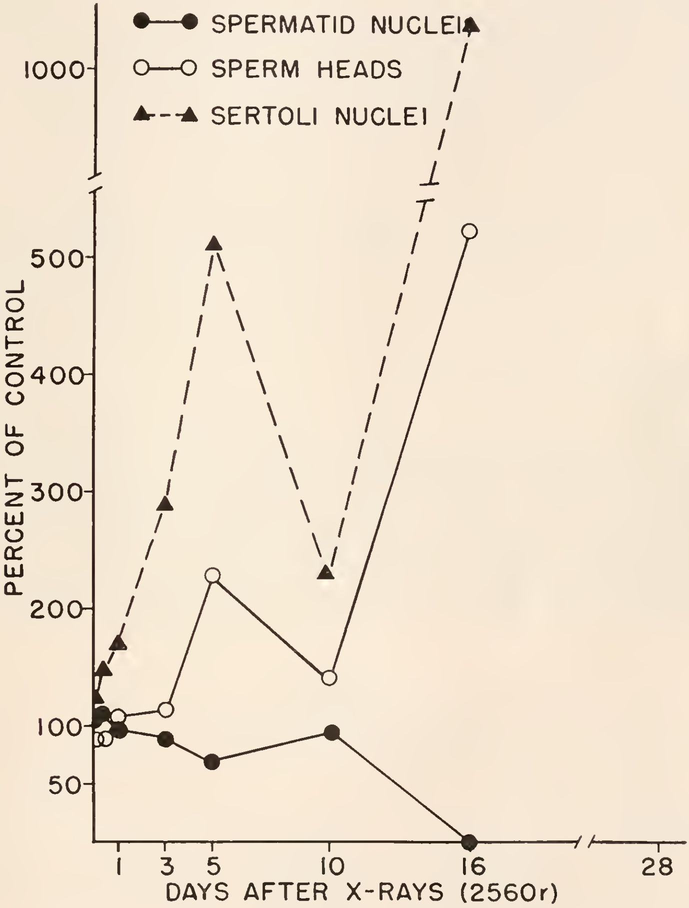


FIGURE 4. Strain S. Incidence of spermatids, sperm and Sertoli nuclei at different times following 2560 r of x-rays.

The data pertaining to the Sertoli cell fraction indicate that the trend is similar in both strains but differs in magnitude. Thus in the case of strain Ba the relative area occupied by this class undergoes a steady increase over the period 0–5 days at which time the level reached is about 2.5 times the control. This value then falls to about 2 times the control value by 10 days, and then increases again reaching at 16 days a level about 17 times the control value. In the case of strain S

TABLE I

Frequency of cell types at various times after 2560 r of x-rays[2]

Stage and strain		1 hour	8 hours	1 day	3 days	5 days	10 days	16 days	28 days
Spermatogonia in interphase	Ba	103.5	72.5	51.6	13.4	7.7	13.3	0.0	0.0
	S	90.1	73.5	29.9	5.5	1.1	8.9	0.0	—
Spermatogonia in mitosis	Ba	30.5	9.7	1.7	5.1	0.0	4.3	0.0	0.0
	S	65.4	41.9	6.9	0.0	0.0	0.0	0.0	—
Spermatocyte chromatin	Ba	105.6	111.4	95.1	128.5	99.2	49.7	0.0	0.0
	S	100.9	100.0	115.1	114.8	79.0	104.0	0.0	—
Spermatid nuclei	Ba	113.5	105.6	110.7	72.4	68.1	138.1	0.0	0.0
	S	106.2	110.2	97.8	91.6	69.7	97.7	0.0	—
Sperm heads	Ba	59.9	88.9	106.3	120.8	222.2	178.2	78.4	4.4
	S	89.8	91.2	107.3	113.5	227.5	139.7	521.6	—
Sertoli nuclei	Ba	97.9	107.6	181.6	223.2	250.1	212.5	1,702	1,860
	S	121.6	143.6	172.1	293.4	510.5	227.2	1,036	—

[2] Values expressed as per cent of controls.

the corresponding values are at 5 days 5 times, and at 10 days 2.2 times the control levels. The value attained by 16 days is about 11.4 times the control level. The cause of this striking difference between the strains, at 5 days, is not clear. These changes in relative areas of the Sertoli fraction are, within limits, reflections of changes in area of the spermatogenic cells.

In Table II are presented the data with respect to spermatogonial and non-spermatogonial necrosis. Data from our previous experiment (320 r) are included

TABLE II

Spermatogonial and non-spermatogonial necrosis at various times after 320 r or 2560 r of x-rays

Strain and time after x-rays		320 r		2560 r	
		% spermatogonial necrosis	% non-spermatogonial necrosis	% spermatogonial necrosis	% non-spermatogonial necrosis
Ba	Control	3.8	2.1	3.8	2.1
S		4.0	2.5	4.0	2.5
Ba	1 hour	3.4	3.0	6.0	3.7
S		5.8	2.4	6.5	1.2
Ba	8 hours	9.7	2.2	15.3	3.0
S		8.8	1.7	14.9	2.0
Ba	24 hours	7.4	2.3	0.0	4.5
S		4.6	2.2	0.0	3.2

in this table for purposes of comparison. All data in this table are expressed as percentages.

Changes in area occupied by the spermatogonial fraction (normal interphasic + mitotic + necrotic spermatogonia) following exposure to x-rays are presented in summary form in Table III.

Table III

A comparison of expected and observed frequencies of spermatogonia at various times following exposure to x-rays of different dose levels

Time after x-rays	Strain					
	Ba			S		
	% total spermatogonia	Difference from control	Difference as % of control	% total spermatogonia	Difference from control	Difference as % of control
Control	12.35			15.10		
320 r 1 hour	14.47	+2.12	17.17	13.55	−1.55	10.26
8 hours	9.89	−2.46	19.92	12.29	−2.81	18.61
24 hours	10.39	−1.96	15.87	6.78	−8.32	55.10
2560 r 1 hour	9.11	−3.24	26.23	13.21	−1.79	11.85
8 hours	10.27	−2.08	16.84	11.61	−3.49	23.11
24 hours	3.32	−9.03	73.12	3.01	−12.09	80.07

Discussion

In the case of the testis it is clear that any treatment which interferes with, or prevents, spermatogonial mitotic activity will bring about partial or complete maturation depletion of the seminiferous tubules. Temporary depletion will follow if, for a short period of time, the level of spermatogonia is reduced much below normal thereby preventing the quantitative replacement of the spermatocyte fraction. Such a reduction may be brought about either by inhibition of chromosomal reduplication (and therefore of mitosis), a relatively high level of spermatogonial necrosis or by some combination of these. Depletion of the permanent type may be brought about in the same manner but with the added proviso that spermatogonial regeneration must also be prevented.

There is ample evidence on hand that exposure to x-rays brings about the onset of maturation depletion (see introduction for references). It then follows that the effects of a dose of x-rays large enough to produce a permanent absence of spermatogonia must differ quantitatively and/or qualitatively from the effects of a dose causing only temporary changes. The present work is concerned with the response of the mouse testis to a dose of x-rays large enough to induce permanent depletion of the seminiferous tubules.

In order to facilitate comparison with the present results, a brief resumé of previous results utilizing a dose of x-rays of 320 r (Bryan and Gowen, 1956) is included here. With respect to interphasic spermatogonia, our 320 r data indicate a marked decline to less than 10% of the control value by three days following exposure. This low level remains in effect until 10 days, at which time regeneration commences. Spermatogonial mitotic activity follows a different course. There is an initial decline reaching a low point 8 hours after exposure, then a marked rise during the 8–24-hour period. This is followed by a further and more extensive decline during 1–3 days post-irradiation. Thereafter the pattern of response is essentially the same as for the non-dividing cells. The 2560 r data show marked deviations from this pattern. The area occupied by interphasic spermatogonia undergoes a rapid decline reaching a very low level by 5 days. There is a slight rise during the 5–10-day period, but the level then declines to zero by 16 days. The mitotic spermatogonia follow a similar pattern but the rate of decline during the early post-irradiation period (1–24 hours) is much more rapid. There is, then, no rise in mitotic activity during the 8–24-hour period; nor does repopulation of the seminiferous tubules take place. These facts lend themselves to the interpretation, that following exposure to 2560 r of x-rays, spermatogonial mitosis must be delayed or inhibited for a longer period of time than in the case of the 320 r experiment. Furthermore the surviving spermatogonia must be unable to sustain a regenerative phase after the initial inhibitory effects of the irradiation have worn off.

As stated above, spermatogonial necrosis may contribute to some extent to the depletion process. It is also to be expected that large doses of x-rays would be likely to produce a greater frequency of cell death than small doses. Hence it is probable that spermatogonial cell death may play a more prominent role in the initiation of maturation depletion following exposure to large doses of x-rays. With these points in mind, the pertinent sections were analyzed (by the Chalkley method) for spermatogonial and non-spermatogonial necrosis. These data are listed in Table II together with corresponding data obtained from the 320 r experiment. Exposure to either dose of x-rays increases the frequency of necrosis above control levels by one hour after irradiation. Similarly, a peak is reached at the 8-hour period followed by a return to lower levels at 24 hours. The pattern of response is therefore about the same for either dose of x-rays; however, there is a difference in the magnitude of this response. At 8 hours after irradiation with 2560 r, the frequency of spermatogonial necrosis is almost double that found following exposure to 320 r. Then at 24 hours following exposure no cells were encountered which could be classified as necrotic spermatogonia. These observations may be interpreted in two ways. On the one hand it may mean that a large fraction of cells are heavily damaged and undergo degenerative changes even though they are far removed, in time, from mitotic activity. The remaining cells, then, constitute a less severely damaged fraction which may undergo degeneration with the onset of mitosis. This interpretation is in accord with the views of Lasnitski (1943) concerning the effect of large doses of x-rays (2500–10,000 r). The possibility also exists that the observed absence of necrotic spermatogonia is correlated with the very low mitotic rate 24 hours following exposure. This view receives some support from the reports of Glücksmann and Spear (1939) and others, to the effect that irradiation-induced cellular degeneration occurs at about the same time as the onset of mitotic activity. Then it follows that under conditions where mitotic levels are low (as in the present case) the chances of encountering necrotic cells are likewise much reduced. It is obvious from the present data that spermatogonial nuclei survive for different periods of time (some are present at 5–10 days following exposure). This must mean that some cells have suffered less damage than others, yet this fraction also is destined to be eliminated from the tubules by 16 days following x-ray exposure. From this we may conclude that severely damaged cells may undergo degeneration prior to the onset of mitosis (in agreement with Lasnitski), while less severely damaged cells do not degenerate until they attempt to enter mitosis.

With respect to the foregoing discussion, the relations between the levels of necrosis induced by different doses of x-rays are of significance. As Table II shows, an 8-fold increase in dose approximately doubles the frequency of necrotic cells at the 8-hour period. Although the conditions of the present experiments are quite different from those of Lasnitski (1943), who used tissue cultures of chick fibroblasts, nevertheless the results are fairly similar. This author's results indicate that a four-fold increase in dose increased the frequency of necrosis to about 1.4 times that of the low dose, whereas a 1.1-times increase in necrosis resulted when the dose was doubled. If a curve is fitted to these data it is found that an 8-fold increase in dose should result in the approximate doubling of the necrotic level (as observed in the present work). This offers further support for the idea that, as the x-ray dose is increased, cells further removed from the sensitive period are likely to suffer lethal injury.

It may be argued that fixed and stained preparations do not allow a very accurate estimation of the frequency of necrosis. Unless the intervals between the fixation times are less than the time necessary for cells to undergo lysis and be eliminated, such estimates are likely to be minimum values. However this is open to verification. Thus from control data the total area of the tubules occupied by spermatogonia (normal + necrotic) can be determined. A comparison of this value with similar determinations on irradiated material will reveal the goodness of fit existing between the expected and observed frequencies. The important point is whether or not any irradiation-induced decrease in area occupied by normal spermatogonia can be accounted for by an increase in the necrotic value. An analysis of this kind is summarized in Table III. It can be seen that, with the exception of the Ba 320 r, one-hour material, each time period shows a deficiency of spermatogonia. Following exposure to 320 r, these deficiencies range from about 10% to 20% of control values during the first 8 hours. With respect to the 2560 r experiment, the corresponding values range from about 12% to 26% of controls. It is clear that these changes in area are, during the first 8 hours, quite similar despite the difference in dose levels employed. Since the data in Table III take into account spermatogonial necrosis, the observed deficiencies cannot be accounted for on the basis of the increased frequency of necrosis as reported in Table II.

On *a priori* grounds it would be logical to impute the observed difference to additional and "unobserved" spermatogonial necrosis. This is not an entirely satisfactory explanation. The stage at which cells are most sensitive to irradiation corresponds to that portion of the mitotic cycle during which chromosomal reduplication is taking place. Irradiation does not inhibit DNA synthesis in cells which are already in the period of synthesis, but delays these cells in entering division (see Howard and Pelc, 1953). At the time of irradiation, then, there is a fraction of spermatogonia which has passed the critical stage. These cells are most probably those observed in mitosis during the first few hours following exposure. In confirmation of this are the results of Bullough and Van Oordt (1950) and others, which indicate that, in the mouse, the duration of mitosis (prophase–telophase) is of the order of three hours. Now a certain proportion of these dividing spermatogonia transform into spermatocytes. These products of division are, therefore, lost from the spermatogonial fraction. Thus it follows that shortly after irradiation, the spermatogonial fraction will be decreased both by loss of these cells and by the reduction in frequency of replacement divisions. Unfortunately it is not possible to calculate the decrease in the spermatogonial fraction to be expected on these grounds. However it is evident that the deficiencies reported in Table III cannot entirely be ascribed to "unobserved" necrosis. The estimates of necrosis as determined in the present work are, therefore, reliable indices of irradiation-induced cellular degeneration.

The effects described above are cumulative, and therefore any deficiency should become progressively more marked with time following exposure to large doses of x-rays. Reference to Table III shows that this is the case. Exposure to 2560 r results in reduction of spermatogonial area to 20–27% of control values by 24 hours. In addition, it was expected that the 2560 r data would show trends similar to those following exposure to 320 r, but of greater magnitude. The data of Tables II and III are in agreement with this, but the relation is somewhat obscured by variation in the level of spermatogonia scored at 24 hours following exposure to 320 r. Since relatively few animals were used in these experiments, sampling errors undoubtedly contribute to this observed variation between strains.

In the case of the 320 r experiment, the surviving fraction of spermatogonia eventually repopulate the tubules. This suggests that recovery has occurred prior to the onset of mitotic activity 10 days following exposure. It is also possible to interpret these findings to mean that the surviving spermatogonia constitute a relatively more resistant fraction. Such an interpretation would be in accord with the conclusions of Eschenbrenner *et al.* (1948). In marked contrast are the results of the 2560 r experiment. Here, also, a small fraction of spermatogonia are present at 5 days following exposure. At 10 days, both strains show a slight increase in spermatogonia over the 5-day levels, but by 16 days, spermatogonia have been completely eliminated. These observations may be explained by the assumption of a further period of necrosis during the 10–16-day interval. This implies, in contrast to the 320 r case, that the spermatogonia present at 5 days subsequently attempt to undergo mitosis at which time latent damage expresses itself.

The spermatogonia present at 5 days following exposure to either dose of x-rays have the morphological characteristics of the so-called type A or "dusty" spermatogonia. Type A cells are regarded as "stem-line" germ cells by Clermont and Leblond (1953). These authors point out that a small fraction of Type A spermatogonia after one division cycle become "dormant." Such "dormant" cells do not divide again until later in the spermatogenic cycle. In other words these spermatogonia remain "dormant" for about 6 days following the initiation of this inactive phase. This means that, in these cells, recovery from the effects of radiation exposure should be possible before mitosis recommences. With respect to our 320 r material, this apparently is the case. On the other hand, any recovery following exposure to 2560 r must only be partial since repopulation does not occur —despite the suggestion of an increase in frequency of spermatogonia by 10 days post-irradiation. On these grounds the spermatogonia present at 5 days following exposure to 2560 r (some or all of which may in fact be "dormant" type A cells) must be capable of limited division. Otherwise, the frequency of spermatogonia would not undergo the changes observed during the 5–16 day period.

Further evidence which points up the more widespread damage produced by exposure to this high dose of x-rays is provided by a consideration of non-spermatogonial necrosis. Reference to Table II shows that at 24 hours, the necrotic level has risen to 1.5–2.0 times that of the control. This increase can be largely accounted for on the basis of the very high frequency of degenerating metaphase I spermatocytes. It was observed that practically all metaphase I plates present in sections of these tubules were necrotic. In the 320 r experiment, the frequency of non-spermatogonial necrosis remained close to control levels. Very few necrotic metaphase I spermatocytes were encountered in this material.

The present data, when considered together with the results of our 320 r experiment, allow several conclusions to be drawn with respect to the manner in which the histological effects of radiation exposure are brought about. Certain of these conclusions take on added significance when considered in the light of other experimental approaches. Cells are prevented from entering division following irradiation. This may come about either through inhibition of DNA synthesis (chromosomal reduplication) or by death of the cells. Both mechanisms play a role in the irradiation-induced depletion of spermatogonia. The death of cells plays a more important role in this process following high doses of irradiation—such as 2560 r—than following exposure to low doses (320 r in the present case). Irradiation-induced mitotic inhibition would appear to be the major factor following exposure to relatively low doses of x-rays. As is readily apparent, this effect of x-rays on cells is of a very basic nature. It must perforce be taken into consideration if the nature of the effects of irradiation on biological systems is to be evaluated in a proper manner.

It is clearly evident that different levels of injury are produced by the irradiation treatments used here. Thus following 320 r, the surviving spermatogonial fraction is capable of mitotic activity to the extent necessary for the initiation of tubule repopulation. After exposure to 2560 r, on the other hand, the survivors are incapable of such a sustained effort.

SUMMARY

1. Changes in the cellular composition of the seminiferous tubules induced by exposure to 2560 r of x-rays have been analyzed by a quantitative histological procedure. These data have been compared with the results obtained following ex-

posure to a much lower dose (320 r) in an attempt to gain further insight with respect to the manner in which the observed changes are brought about.

2. Exposure to 320 r results in a temporary maturation depletion of the seminiferous epithelium. This is brought about mainly by the inhibition of spermatogonial mitosis with irradiation-induced spermatogonial necrosis playing only a minor role. In contrast, exposure to 2560 r produces a permanent depletion due to the fact that surviving spermatogonia are incapable of sustained regenerative efforts.

3. The frequency of necrotic spermatogonia, following 2560 r, was found to be double the peak value attained in the 320 r material, or four times that of the corresponding controls.

4. Taken together, the data for the 320 r and 2560 r experiments suggest that spermatogonial depletion is brought about in two ways: (1) by suppression of mitosis due to inhibition of DNA synthesis, and (2) the killing of cells. Irradiation-induced necrosis plays a much more important role following exposure to high doses of x-rays. Even so the frequency of necrosis in either experiment did not reach very high levels, being about 9% after the low dose and about 15% in the case of the high x-ray dose.

5. Further evidence was obtained in support of the view that relatively heavily damaged cells may undergo degenerative changes prior to the onset of division, while less heavily damaged cells manifest degenerative changes only at about the time of entry into mitosis.

LITERATURE CITED

Bryan, J. H. D., and J. W. Gowen, 1956. A histological and cytophotometric study of the effects of x-rays on the mouse testis. *Biol. Bull.*, **110**: 229–242.

Bullough, W. S., and G. J. Van Oordt, 1950. The mitogenic actions of testosterone propionate and oestrone on the epidermis of the adult male mouse. *Acta Endocrinol.*, **4**: 291–305.

Chalkley, H. W., 1943. Method for quantitative morphologic analysis of tissues. *J. Nat. Cancer Inst.*, **4**: 47–53.

Clermont, Y., and C. P. Leblond, 1953. Renewal of spermatogonia in the rat. *Amer. J. Anat.*, **93**: 475–501.

Eschenbrenner, A. B., E. Miller and E. Lorenz, 1948. Quantitative histologic analysis of the effect of chronic whole-body irradiation with gamma rays on the spermatogenic elements and the interstitial tissues of the testes of mice. *J. Nat. Cancer Inst.*, **9**: 133–148.

Eschenbrenner, A. B., and E. Miller, 1950. Effect of roentgen rays on the testis: Quantitative histological analysis following whole-body exposure of mice. *Arch. Pathol.*, **50**: 736–749.

Forssberg, A., and G. Klein, 1954. Studies on the effects of x-rays on the biochemistry and cellular composition of ascites tumors. II. Changes in the pattern of glycine-2-C^{14} incorporation during the first two hours after irradiation *in vivo*. *Exp. Cell Res.*, **7**: 480–497.

Glücksmann, A., and F. G. Spear, 1939. The effect of gamma radiation on cells *in vivo*. Part II. 1. Single exposures of the fasting tadpole at room temperature. 2. Single exposures of the normal animal at low temperature. *Brit. J. Radiol.*, **12**: 486–498.

Hertwig, P., 1938. Die Regeneration des Samenepithels der Maus nach Röntgenbestrahlung, unter besonderer Berucksichtigung der Spermatogonien. *Arch. exp. Zellforsch.*, **22**: 68–73.

Holmes, B. E., 1947. The inhibition of ribo and thymo-nucleic acid synthesis in tumour tissue by irradiation with x-rays. *Brit. J. Radiol.*, **20**: 450–453.

Howard, A., and S. R. Pelc, 1953. Synthesis of desoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. *Heredity,* **6** (Suppl.) : 261–274.

Lasnitski, I., 1943. The response of cells *in vitro* to variations in x-ray dosage. *Brit. J. Radiol.,* **19**: 250–256.

Oakberg, E. F., 1955. Sensitivity and time of degeneration of spermatogenic cells irradiated in various stages of maturation in the mouse. *Radiation Res.,* **2**: 369–391.

Regaud, C. L., and J. Blanc, 1906. Action des rayons X sur les diverses générations de la lignée spermatique. Extrême sensibilité des spermatogonies à ces rayons. *C. R. Soc. Biol.,* **61** : 163–165.

Regaud, C. L., and A. Lacassagne, 1927. Effects histophysiologiques des Rayons de Roentgen et de Becquerel-Curie sur les tissus adultes normaux des animaux supérieurs. *Arch. de l'Institut du Radium,* I, 1 *et seq.*

Shaver, S. L., 1953. X-irradiation injury and repair in the germinal epithelium of male rats. I. Injury and repair in adult rats. *Amer. J. Anat.,* **92**: 391–432.

Smellie, R. M. S., G. F. Humphrey, E. R. M. Kay and J. N. Davidson, 1955. The incorporation of radioactive phosphorus into the nucleic acids of different rabbit tissues. *Biochem. J.,* **60**: 177–193.

LARVAL DEVELOPMENT OF BALANUS AMPHITRITE VAR. DENTICULATA BROCH REARED IN THE LABORATORY[1]

JOHN D. COSTLOW, JR. AND C. G. BOOKHOUT

Duke University Marine Laboratory, Beaufort, N. C., and
Department of Zoology, Duke University, Durham, N. C.

While there have been numerous studies on Cirripedia larvae based on reconstructed life-histories, to date there have been only three reports on nauplii reared from the egg through all larval stages to the sessile form, that of Herz (1933) for *Balanus crenatus,* Hudinaga and Kasahara (1941) for *Balanus amphitrite hawaiiensis,* and Costlow and Bookhout (1957) for *Balanus eburneus.* This method has the advantage over reconstructed life-histories in that one can be sure of the identity of the adult, the source of eggs and future larvae. Once the life-histories of all species of barnacles in a given area have been described, ecological studies may be made with a greater degree of assurance. Ecological investigations based on sampling, such as that of Bousfield (1955), may provide the number of stages, the approximate duration and mortality of the individual stages, and the distribution and fluctuations in large populations. Laboratory studies on individually reared larvae, however, can give more detailed information on all phases of the life-history other than distribution and population fluctuations. Both types of research are required before a complete picture can be obtained. Laboratory studies are necessarily prerequisite to physiological and genetic investigations.

Balanus amphitrite denticulata Broch is one of the most widely distributed acorn barnacles. It has been reported from the tidal waters of Britain which are artificially heated by industrial effluents (Crisp and Molesworth, 1951), the estuaries of South Africa (Sandison, 1954), and the East and West coasts of North America (Dr. Dora Henry, personal communications). In spite of its world-wide distribution only the first two larval stages have been described (Sandison, 1954). Bishop (1950) believes that *Balanus amphitrite denticulata* merits a more thorough study and questions its position as a variety of *B. amphitrite.* Thus it should be of interest to compare the larval development of this variety with the descriptions of larvae of *Balinus amphitrite albicostatus* (Ishida and Yasugi, 1937) and *Balanus amphitrite hawaiiensis* '(Hudinaga and Kasahara, 1941).

Balanus amphitrite denticulata is the most abundant fouling organism in the inter-tidal region at Beaufort, North Carolina and breeds during the same summer months as *Balanus eburneus.* During the past two years we have followed the larval development of *B. amphitrite denticulata* in the laboratory to determine the number of stages, the frequency of molting, and the duration of the intermolt periods. Our secondary objectives were to compare the appendage setation and body form with the corresponding naupliar stages of the other two varieties of *Balanus amphitrite* which have been described and with the larvae of barnacles belonging to different species and genera.

---

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Duke University NR 104-194.

The technique of rearing individual larvae of *Balanus amphitrite denticulata* was identical to that described for *Balanus eburneus* (Costlow and Bookhout, 1957). Mass cultures of the larvae were maintained on a diet of *Chlamydomonas* sp. and fertilized *Arbacia* eggs at a constant temperature of 26° C.

Results and Discussion

The nauplii of *Balanus amphitrite denticulata* reared individually in the laboratory pass through 6 stages and one cyprid stage.

Nauplii. The most significant morphological characteristics of each naupliar stage are given below.

Stage I. (Fig. 1, I.) The curved frontolateral horns project caudo-laterally, are relatively long, and situated close to the carapace. The carapace is rounded and the abdominal process terminates in two short spines (Fig. 1, Ia). All setae are devoid of setules (Fig. 1, Ib, c, d).

Stage II. (Fig. 1, II.) The frontolateral horns project laterally. Anterior to the small lateral spines the carapace has relatively straight lateral borders. Posterior to the spines the carapace tapers into the caudal process which bears a small dorsal and ventral tooth at its midpoint (Fig. 1, IIa). The abdominal process bears one spine on each side of the base and the maxillules are prominent (Fig. 1, IIa). The majority of the setae now bear setules (Fig. 1, IIb, c, d).

Stage III. (Fig. 1, III.) The frontolateral horns are tapered from a slightly swollen basal portion adjoining the carapace. The lateral edges of the carapace are rounded and the lateral spines of stage II are missing. The abdominal process bears the same spines as in stage II (Fig. 1, IIa).

Stage IV. (Fig. 2, IV.) A pair of short carapace spines marks the posterior edge where the carapace is delimited from the caudal process. Two pairs of spines are located on the abdominal process. The posterior pair is located at the base of the abdominal process and the anterior pair is in line with the division between the caudal and abdominal processes. A pair of small teeth appears just anterior to the bifurcated end of the abdominal process. Six rows of minute bristles are found on the ventral surface of the abdominal process (Fig. 2, IVa).

Stage V. (Fig. 2, V.) The anterior and posterior pairs of spines of the abdominal process remain as in stage IV. Lateral to the posterior abdominal spine, however, a smaller spine is added on each side. The sub-terminal teeth and bristles remain as in the previous stage (Fig. 2, Va).

Stage VI. (Fig. 2, VI.) Six pairs of small spines on the ventral surface of the abdominal process replace the bristles of the previous stage. The developing cirriform appendages are visible *beneath* the exoskeleton, but are not shown in Figure 2, VIa. The anterior pairs of spines, found in stage V, are not present but the small paired lateral spines and large paired median spines remain. Paired eyespots develop in the later sixth stage nauplii.

Cyprid. The rounded anterior end of the cyprid is the widest portion of the carapace, curving gradually to the posterior end. The degree of pigmentation varies considerably but some brown pigment was always observed. When the bivalve carapace is closed both the anterior and posterior ends are smooth.

The number of naupliar stages observed for *B. amphitrite denticulata* is consistent with our findings for *Balanus eburneus* (Costlow and Bookhout, 1957) and

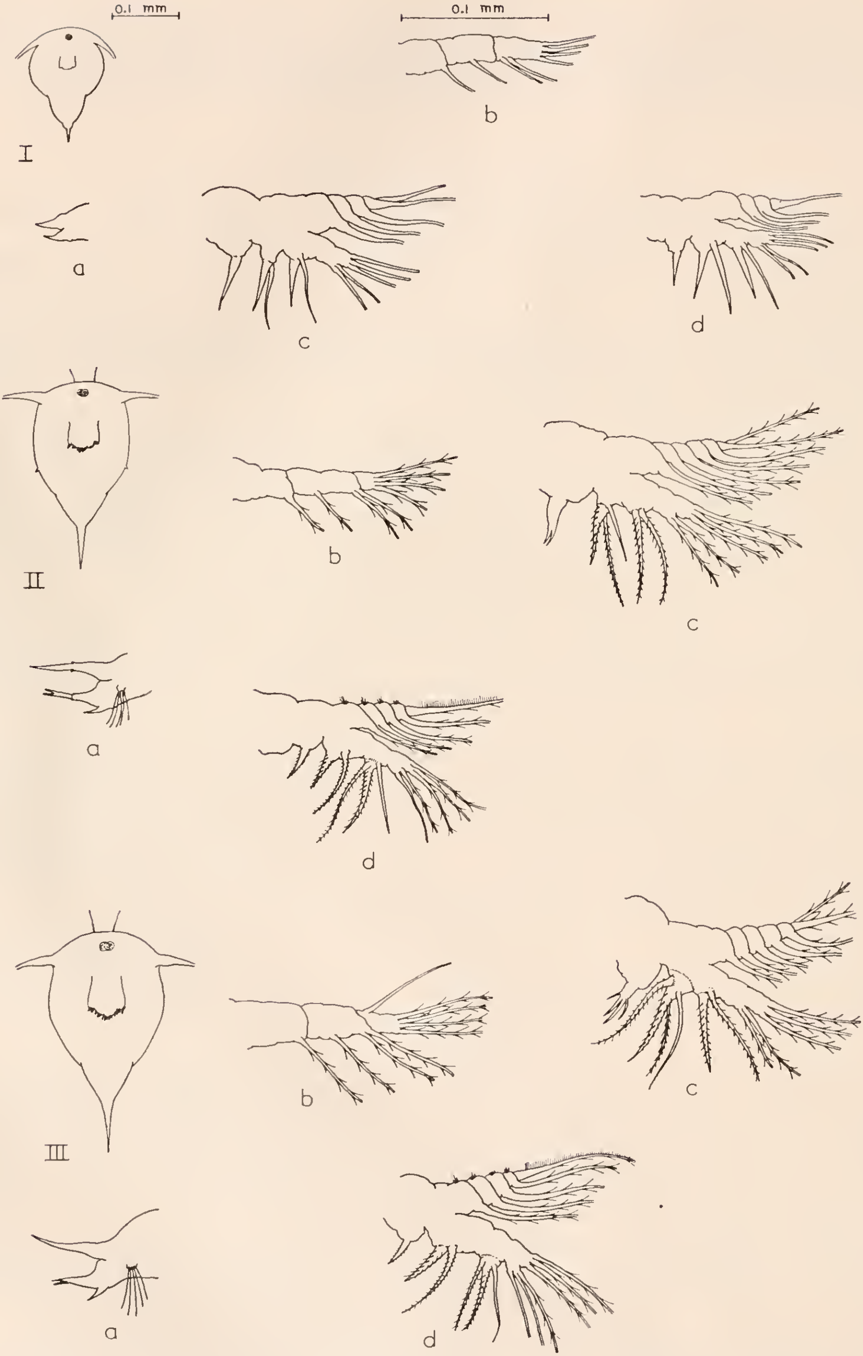


FIGURE 1. Carapace, caudal and abdominal processes, and appendages of naupliar stages I, II, and III of *Balanus amphitrite denticulata* reared in the laboratory. All swimming setae are cut short. a, lateral view of abdominal and caudal processes; b, antennule; c, antenna; d, mandible.

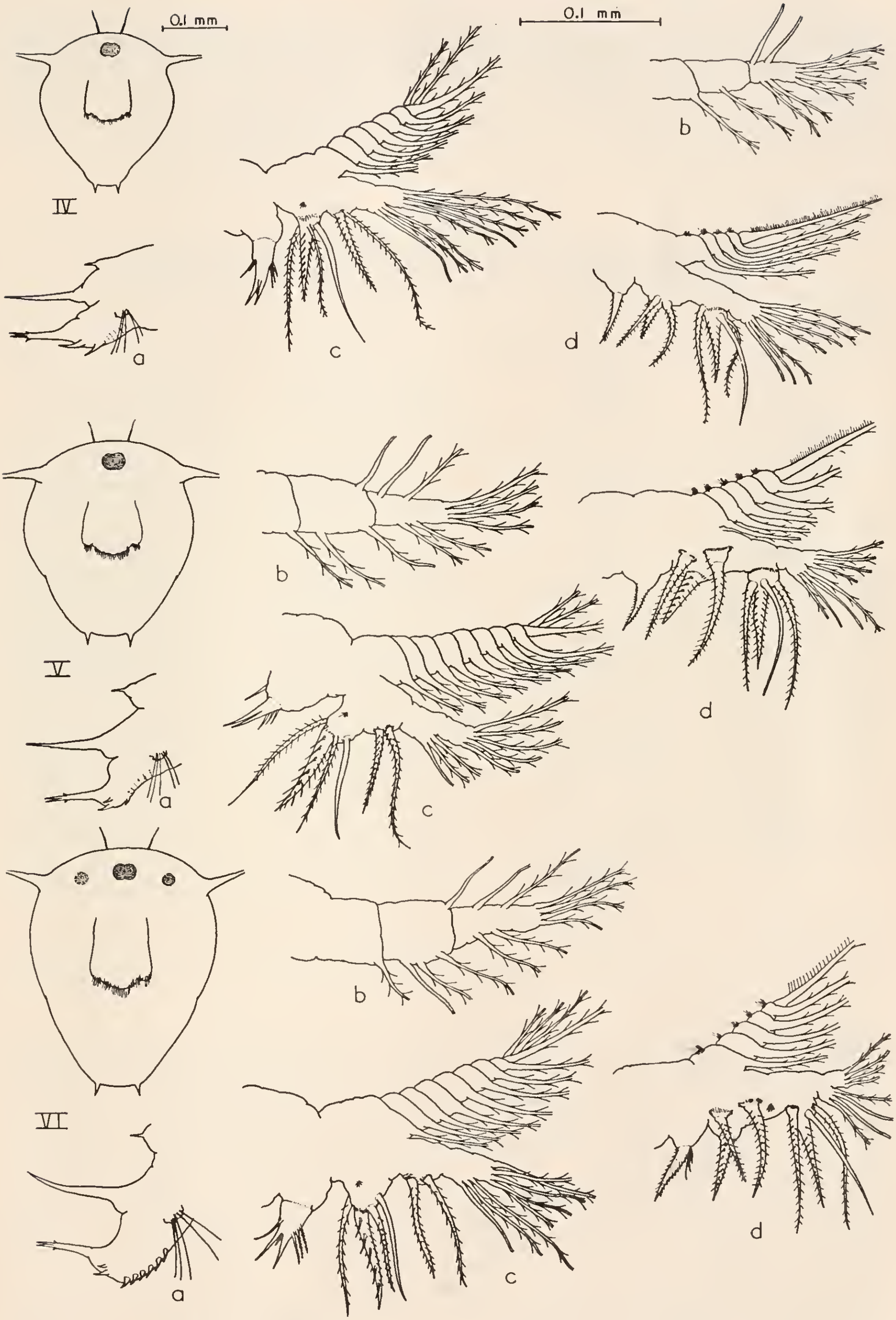


FIGURE 2. Carapace, caudal and abdominal processes, and appendages of naupliar stages IV, V, and VI of *Balanus amphitrite denticulata* reared in the laboratory. All swimming setae are cut short. a, lateral view of abdominal and caudal processes; b, antennule; c, antenna; **d**, mandible.

for numerous species whose larval stages have been reconstructed from planktonic material. The seventh naupliar stage described for *Balanus amphitrite albicostatus* by Ishida and Yasugi (1937) and for *Balanus amphitrite hawaiiensis* by Hudinaga and Kasahara (1941) differed from the sixth stage only in the presence of completely developed paired eyes. In *B. amphitrite denticulata*, as in *B. eburneus*, the development of paired eyes occurs within the sixth naupliar stage.

Table I

Comparison of appendage setation for nauplii of B. amphitrite denticulata (B.a.d.), B. amphitrite albicostatus (B.a.a.) and B. amphitrite hawaiiensis (B.a.h.)

Stage		Antennule	Antenna	Mandible
I	*B.a.d.*	0.4.2.1.1.	0.2.3.–0.3.2.2.2.G.	0.1.3.–0.3.2.2.2.G.
	B.a.a.	0.4.2.1.–	0.1.4.–0.3.2.2.2.G.	0.1.3.–0.3.2.3.2.G.
	B.a.h.	0.4.2.1.1.	0.2.3.–0.3.2.2.3.G.	0.1.3.–0.3.2.3.3.G.
II	*B.a.d.*	0.4.2.1.1.	0.2.5.–0.3.2.2.3.G.	0.1.4.–0.3.2.3.2.G.
	B.a.a.	0.4.2.1.1.	0.1.6.–0.3.2.2.2.G.	0.1.3.–0.3.2.3.2.G.
	B.a.h.	0.4.2.1.1.	0.2.5.–0.3.2.2.3.G.	0.1.4.–0.3.2.3.3.G.
III	*B.a.d.*	1.4.2.1.1.	0.2.5.–0.3.2.2.4.G.	0.1.4.–0.3.3.3.2.G.
	B.a.a.	1.4.2.1.1.	0.1.6.–0.3.2.2.3.G.	0.1.3.–0.4.2.3.2.G.
	B.a.h.	1.4.2.1.1.	0.2.5.–0.3.2.2.4.G.	0.1.4.–0.3.3.3.3.G.
IV	*B.a.d.*	1.1.4.2.1.1.	0.3.6.–0.5.3.2.4.G.	0.1.4.–0.4.3.4.3.G.
	B.a.a.	1.1.4.2.1.1.	0.2.7.–0.4.3.2.3.G.	0.1.4.–0.4.2.3.3.G.
	B.a.h.	1.1.4.2.1.1.	0.3.6.–0.5.3.2.4.G.	0.1.4.–0.4.3.3.3.G.
V	*B.a.d.*	1.1.1.4.2.1.1.1.	0.3.8.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.
	B.a.a.	1.1.1.4.2.1.1.1.	0.2.9.–0.4.3.2.3.G.	0.1.5.–0.4.2.3.3.G.
	B.a.h.	1.1.1.4.2.1.1.1.	0.3.8.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.
VI	*B.a.d.*	1.1.1.4.2.1.2.1.	0.4.8.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.
	B.a.a.	1.1.1.4.2.1.2.1.	0.3.9.–0.5.3.2.4.G.	0.1.5.–0.4.2.4.4.G.
	B.a.h.	1.1.1.4.2.1.2.1.	0.3.9.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.
VII	*B.a.d.*	none	none	none
	B.a.a.	1.1.1.4.2.1.2.1.	0.3.9.–0.5.3.2.4.G.	0.1.5.–0.4.2.4.4.G.
	B.a.h.	1.1.1.4.2.1.2.1.	0.3.9.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.

Since the introduction of setation formulae for appendages of barnacle nauplii by Bassindale (1936) all investigators have maintained that setation alone was not a reliable criterion for separating larval stages of different species. Some investigators believe it is a useful criterion when used with other morphological characters, such as length of whole body, length and width of carapace, and body spine structure. Others believe it is merely a developmental feature which is of no value in separating larvae. Nevertheless, most authors have continued to give the setation formulae for the naupliar stages described. Knight-Jones and Waugh (1949) summarized the setation formulae for the first two naupliar stages of seven species of barnacles. They conclude that small differences and similarities in setation during the early stages are of no systematic significance and may be-used only as secondary criteria for identification of stages of a given species. In order to evaluate whether setation formulae are significant or not, even as secondary characters, we believe it would be helpful to first determine if the formulae of closely related varieties of *B. amphitrite* are identical or different. If they prove to be different, and useful in separating varieties, they may well be used to separate species as well as genera.

Table I gives the appendage setation for each stage of *B. amphitrite denticulata* and compares this variety with *B. amphitrite hawaiiensis* and *B. amphitrite albicostatus.* The antennule setation is identical for each corresponding stage of all three varieties but can be used to determine whether one is examining stage III, IV, V or VI. Using the setation of the antenna and mandible, however, all stages of the three varieties other than the fifth of *B. amphitrite hawaiiensis* and *B. amphitrite denticulata* can be separated from one another with the setation of the mandible being more consistently different than that of the antenna.

Table II gives the other species of *Balanus,* as well as species of other genera, which have the same setation of individual appendages as *B. amphitrite denticulata.* Setation formulae from the available literature indicate that when all three pairs of appendages are considered, the first stage of *B. amphitrite denticulata* can be separated from all other species described. For example, *B. amphitrite albicostatus* has identical antennule and antenna setations but the mandible is different (Table II). Stage II of *B. amphitrite denticulata* is identical in setation of all appendages to *Balanus improvisus, Chthamalus dentatus,* and *Octomeris angulosa* as shown in Table II by the appearance of these three species in all three columns. While the third stage may be separated from all other species by setation, the fourth stage of *B. amphitrite denticulata* is identical to the fourth stage of *Balanus trigonus.* The fifth naupliar stage has setation identical to *B. amphitrite hawaiiensis, B. eburneus,* and *B. improvisus,* while the sixth stage nauplius is identical only to *B. improvisus* (Jones and Crisp, 1954). Thus, in areas where two or more of these species of barnacles are associated and there is an overlapping of the breeding season, setation could not be relied upon as the only criterion for identification. It is significant, however, that after the second naupliar stage, identical setation of all appendages is confined to the genus *Balanus.*

In *B. eburneus* (Costlow and Bookhout, 1957), as in *Balanus crenatus* (Pyefinch, 1949) the setation of each individual stage was found to be consistent. This was true also of *B. amphitrite denticulata* and the variability described by Norris *et al.* (1951) for second stage *Balanus improvisus* and *B. amphitrite denticulata* was not observed. Sandison (1954) gives the setation formulae for the appendages of the first two stages of *B. amphitrite denticulata.* Our findings differ from Sandison's only in the antennary exopodite of the second stage. Sandison (1954) gives 0.2.5—0.3.2.2.2.G. whereas we found 0.2.5—0.3.2.2.3.G.

Another character which has proved to be useful when combined with other criteria is carapace width and length and total length, although the latter is less reliable. In cases where setation is identical, marked differences in carapace dimensions may be useful in identification. When dimensions are similar or overlap, however, as they do for *B. amphitrite denticulata* and *B. eburneus* (Table III), this character cannot be used. Both of these species have been reared on *Chlamydomonas* sp. and *Arbacia* eggs at 26° C. in a continuously lighted culture cabinet.

TABLE II

Setation of B. amphitrite denticulata nauplii and other species which have identical setation in one or more appendages

Stage	Antennule	Antenna	Mandible
I	0.4.2.1.1. *B. a. denticulata* *B. a. albicostatus* *B. a. hawaiiensis* *B. algicola* *B. balanoides* *B. crenatus* *B. eburneus* *B. improvisus* *B. perforatus** *B. trigonus* *C. dentatus* *E. modestus* *M. mitella* *O. angulosa* *T. serrata* *T. squamosa* *V. stroemia*	0.2.3.–0.3.2.2.2.G. *B. a. denticulata* *B. a. albicostatus*	0.1.3.–0.3.2.2.2.G. *B. a. denticulata* *B. algicola* *B. crenatus* *B. eburneus* *B. improvisus* *B. perforatus* *B. trigonus* *C. dentatus* *C. stellatus* *E. modestus* *M. mitella* *O. angulosa* *T. serrata* *T. squamosa* *V. stroemia*
II	0.4.2.1.1. *B. a. denticulata* *B. a. albicostatus* *B. a. hawaiiensis* *B. algicola* *B. balanoides* *B. crenatus* *B. eburneus* *B. improvisus* *B. perforatus* *B. trigonus* *C. dentatus* *C. stellatus* *E. modestus* *M. mitella* *O. angulosa* *T. serrata* *T. squamosa* *V. stroemia*	0.2.5.–0.3.2.2.3.G. *B. a. denticulata* *B. a. hawaiiensis* *B. improvisus* *B. perforatus* *B. trigonus* *C. dentatus* *O. angulosa*	0.1.4.–0.3.2.3.2.G. *B. a. denticulata* *B. algicola* *B. improvisus* *C. dentatus* *O. angulosa* *T. serrata*
III	1.4.2.1.1. *B. a. denticulata* *B. a. hawaiiensis* *B. a. albicostatus* *B. algicola* *B. eburneus* *B. improvisus* *B. perforatus* *B. trigonus* *C. stellatus* *E. modestus* *M. mitella* *O. angulosa* *T. squamosa* *V. stroemia*	0.2.5.–0.3.2.2.4.G. *B. a. denticulata* *B. a. hawaiiensis* *B. improvisus* *B. perforatus* *B. trigonus* *O. angulosa*	0.1.4.–0.3.3.3.2.G. *B. a. denticulata*

TABLE II—*Continued*

Stage	Antennule	Antenna	Mandible
IV	1.1.4.2.1.1. *B. a. denticulata* *B. a. albicostatus* *B. a. hawaiiensis* *B. algicola* *B. balanoides* *B. eburneus* *B. improvisus* *B. perforatus* *B. trigonus* *C. stellatus* *E. modestus* *M. mitella* *O. angulosa* *T. squamosa*	0.3.6.–0.5.3.2.4.G. *B. a. denticulata* *B. a. hawaiiensis* *B. trigonus*	0.1.4.–0.4.3.4.3.G. *B. a. denticulata* *B. improvisus* *B. trigonus*
V	1.1.1.4.2.1.1.1. *B. a. denticulata* *B. a. albicostatus* *B. a. hawaiiensis* *B. algicola* *B. eburneus* *B. improvisus* *B. perforatus* *B. trigonus* *O. angulosa*	0.3.8.–0.5.3.2.4.G. *B. a. denticulata* *B. a. hawaiiensis* *B. eburneus* *B. improvisus* *E. modestus*	0.1.5.–0.4.4.4.3.G. *B. a. denticulata* *B. a. hawaiiensis* *B. eburneus* *B. improvisus* *B. perforatus* *E. modestus*
VI	1.1.1.4.2.1.2.1. *B. a. denticulata* *B. a. albicostatus* *B. a. hawaiiensis* *B. algicola* *B. crenatus* *B. eburneus* *B. improvisus* *B. trigonus* *B. perforatus* *O. angulosa*	0.4.8.–0.5.3.2.4.G. *B. a. denticulata* *B. eburneus* *B. improvisus* *E. modestus*	0.1.5.–0.4.4.4.3.G. *B. a. denticulata* *B. a. hawaiiensis* *B. improvisus* *B. perforatus* *B. trigonus* *E. modestus*

* Norris and Crisp, 1954. All other sources indicated in text.

B—Balanus
C—Chthamalus
E—Elminius
M—Mitella
O—Octomeris
T—Tetraclita
V—Verruca

We have no personal observations on how these larvae compare in size with those from the natural environment. The first two stages of *B. amphitrite denticulata* reared by us at Beaufort are slightly larger than those described by Sandison (1954) from South Africa which in turn were larger than those found by Crisp (Sandison, 1954) in Great Britain.

Body form and spine structure should also be considered with setation and body dimensions. The general body form of all nauplii of the three varieties of

Balanus amphitrite is similar but there are differences in spine structure. The lateral spines of the second stage of *B. amphitrite denticulata* (Fig. 1, II), observed also by Sandison (1954), are not found in *B. amphitrite albicostatus* or *B. amphitrite hawaiiensis*. Ishida and Yasugi (1937) show six pairs of small spines on the ventral surface of the abdominal process in the third stage of *B. amphitrite albicostatus*. These do not appear in *B. amphitrite denticulata* or in *B. eburneus* (Costlow and Bookhout, 1957) until the sixth naupliar stage.

Earlier workers introduced into general usage the term "metanauplius," apparently referring to the sixth stage barnacle nauplius. Inasmuch as additional functional appendages are not added during the sixth naupliar stage, it cannot correctly be identified as a metanauplius. It is true that the six pairs of cirriform appendages appear during the sixth stage but they are underneath the exoskeleton and do not function until the cyprid stage. In the larval Cirripedia described to date no true metanaupliar stage has been described.

TABLE III

Measurements of larval stages of B. amphitrite denticulata (B.a.d.) and B. eburneus (B.e.) reared under laboratory conditions at 26° C.

Stage	Carapace				Total length (mm.)	
	Width (mm.)		Length (mm.)			
	B.a.d.	*B.e.*	*B.a.d.*	*B.e.*	*B.a.d.*	*B.e.*
I	0.14–0.20	0.16–0.18	—	—	0.16–0.24	0.19–0.23
II	0.19–0.24	0.21–0.23	—	—	0.25–0.30	0.32–0.34
III	0.22–0.27	0.23–0.27	—	—	0.30–0.34	0.35–0.38
IV	0.25–0.32	0.27–0.32	0.25–0.32	0.30–0.33	0.35–0.39	0.40–0.42
V	0.29–0.36	0.29–0.34	0.30–0.35	0.35–0.38	0.41–0.47	0.44–0.48
VI	0.35–0.43	0.36–0.39	0.35–0.40	0.42–0.50	0.47–0.53	0.54–0.60
C	—	—	—	—	0.49	0.46

The duration of individual stages of *B. amphitrite denticulata* was similar to that found for *B. eburneus* at 26° C. The first stage was short, as in many other species, and lasted from ten minutes to six hours. The duration of the second stage was one day and that of the third stage, one to three days with an average of 1.5 days. Stage IV lasted from one to two days with an average of one day. Stage V averages 1.5 days and stage VI, 2.5 days but with greater variation than in the earlier stages. The molting frequency was similar to that observed for *B. eburneus* (Costlow and Bookhout, 1957).

The size range of *B. amphitrite denticulata* cyprids, 0.45 mm.–0.53 mm., overlaps the range of *B. eburneus* cyprids. Doochin (1951) reported an average length of 0.53 mm. for cyprids of *B. amphitrite niveus* and notes that because of the variation in length it could not safely be used to distinguish cyprids of *B. amphitrite niveus* from those of *B. improvisus*. Barnes (1953) found "at least" two distinct modes in size frequency curves for *B. balanoides* and *B. crenatus* cyprids and interprets them as two populations of distinct sizes, developing in different environments. Pyefinch (1948), noting the discrepancy in *Balanus balanoides* cyprid sizes as reported by Runnström (1925) and Bassindale (1936), suggests that the cyprid attains a greater length in more northern waters. Pyefinch (1948) uses pigmentation and position of eyes to separate the cyprids of *B. balanoides* and *B. crenatus.* Unfortunately, these characters are similar in *B. amphitrite denticulata* and *B. eburneus* cyprids. In both species the anterior end is broader and the dorsal surface curves back to the posterior end. Both posterior and anterior ends are smooth when the bivalve carapace is closed, as in *B. amphitrite niveus,* and the notched appearance of *B. improvisus* cyprids (Doochin, 1951) has not been observed. No definite external characteristic has been found in this study which may be relied upon to differentiate cyprids of *B. amphitrite denticulata* and *B. eburneus.*

The cyprid stage of *B. amphitrite denticulata* could be maintained in the laboratory from one to eight days but successful attachment was observed only in those which settled one to three days following the final naupliar molt. Hudinaga and Kasahara (1941) noted that the settling time of *B. amphitrite hawaiiensis* varied considerably, ranging from a few hours to five days after the final naupliar ecdysis.

The over-all time of development at 26° C. of *B. amphitrite denticulata* from hatching to the settled stage, ranged from seven to ten days, the same time interval observed for *B. eburneus* (Costlow and Bookhout, 1957), when reared at the same temperature. Hudinaga and Kasahara (1941) found that *B. amphitrite hawaiiensis* required seven days at 23–28° C. to attain the cyprid stage and usually one additional day to settle and metamorphose. Ishida and Yasugi (1937) observed that *B. amphitrite albicostatus* attained the cyprid stage in approximately two weeks at 20–25° C. None of their cyprids settled and metamorphosed. Hudinaga and Kasahara (1941) note that *B. amphitrite albicostatus* required only one week for complete development to the young barnacle when fed enough *Skeletonema costatum.* Yasugi (1937) reported that the goose barnacle, *Mitella mitella* L., required nine days for development to the cyprid at 26–28° C. but at 23–26° C. the period was extended to twelve days.

Mortality in *B. amphitrite denticulata* was higher than that of *B. eburneus.* In *B. eburneus* the greatest mortality occurred in the fifth and sixth stages. In *B. amphitrite denticulata* there was no mortality in the fifth stage, 22.2 per cent in the sixth stage, and 49.2 per cent in the cyprid.

While Hudinaga and Kasahara (1941) do not give the approximate number of either *B. amphitrite hawaiiensis* or *B. amphitrite albicostatus* that successfully settled, they do point out that if the food was not adequate, the time for development to the cyprid stage was increased and that survival was reduced. Survival of *B. amphitrite denticulata* under laboratory condition was 12.7 per cent.

Summary and Conclusions

From a study of 126 isolated *Balanus amphitrite denticulata* nauplii reared in the laboratory, plus hundreds in mass culture, the following conclusions may be drawn:

1. The free-swimming larvae of *Balanus amphitrite denticulata* consist of six naupliar stages and one cyprid stage.
2. Carapace size, appendage setation, and spine structure are given for each naupliar stage.

3. Most naupliar stages of *Balanus amphitrite denticulata* may be separated from the larvae of *B. amphitrite albicostatus* and *B. amphitrite hawaiiensis* on the basis of setation formulae; the fifth stage of *Balanus amphitrite denticulata* and *Balanus amphitrite hawaiinensis* have identical setation of all appendages.

4. A comparison of appendage setation with larvae of other species and genera of barnacles described to date indicates that setation formulae are not definitive in distinguishing all stages of all species. Setation is, however, a valuable criterion for identification of nauplii and may be used to separate certain stages and species.

5. At 26° C., the duration of the six naupliar stages of *Balanus amphitrite denticulata* is as follows: first stage, ten minutes to six hours; second stage, one day; third stage, one to three days with an average of 1.5 days; fourth stage, one to two days with an average of one day; fifth stage, one to three days with an average of 1.5 days; and the sixth stage, one to five days with an average of 2.5 days.

6. The duration of the cyprid stage ranges from one to eight days. Successful settling and metamorphosis were observed only in those which settled one to three days after the final naupliar molt.

7. The time required for complete larval development in the laboratory at 26° C. was seven to ten days after hatching. Successful settling and metamorphosis were observed in 12.7 per cent of the 126 nauplii studied under segregated laboratory conditions.

8. Mortality, under laboratory conditions, was highest during the sixth naupliar stage (22.2 per cent) and the cyprid stage (49.2 per cent).

LITERATURE CITED

Barnes, H., 1953. Size variation in the cyprids of some common barnacles. *J. Mar. Biol. Assoc.*, **32**: 297–304.

Bassindale, R., 1936. The developmental stages of three English barnacles, *Balanus balanoides, Chthamalus stellatus* and *Verruca stroemia*. *Proc. Zool. Soc. London*, **106**: 57–74.

Bishop, M. W. H., 1950. Distribution of *Balanus amphitrite* Darwin var. *denticulata* Broch. *Nature*, **165**: 409.

Bousfield, E. L., 1955. Ecological control of the occurrence of barnacles in the Miramichi estuary. National Museum of Canada, Bulletin 37.

Costlow, John D., Jr., and C. G. Bookhout, 1957. Larval development of *Balanus eburneus* in the laboratory. *Biol. Bull.*, **112**: 313–324.

Crisp, D. J., and A. H. N. Molesworth, 1951. Habitat of *Balanus amphitrite* var. *denticulata* in Britain. *Nature*, **167**: 489.

Doochin, H. D., 1951. The morphology of *Balanus improvisus* Darwin and *Balanus amphitrite niveus* Darwin during initial attachment and metamorphosis. *Bull. Mar. Sci. Gulf and Caribbean*, **1**: 15–39.

Herz, L. E., 1933. The morphology of the later stages of *Balanus crenatus* Bruguiere. *Biol. Bull.*, **64**: 432–442.

Hudinaga, M., and H. Kasahara, 1941. Larval development of *Balanus amphitrite hawaiiensis*. *Zool. Mag. (Japan)*, **54**: 108–118.

Ishida, S., and R. Yasugi, 1937. Free-swimming stages of *B. amphitrite albicostatus*. *Botany and Zool., Tokyo*, **V**: 1659–1666.

Jones, L. W. G., and D. J. Crisp, 1954. The larval stages of the barnacle *Balanus improvisus* Darwin. *Proc. Zool. Soc. London*, **123**: 765–780.

Knight-Jones, C. W., and D. Waugh, 1949. On the larval development of *Elminius modestus* Darwin. *J. Mar. Biol. Assoc.*, **28**: 413–428.

Norris, E., L. W. G. Jones, T. Lovegrove and D. J. Crisp, 1951. Variability in larval stages of Cirripedes. *Nature*, **167**: 444–445.

Norris, E., and D. J. Crisp, 1954. The distribution and planktonic stages of the Cirripede *Balanus perforatus* Bruguière. *Proc. Zool. Soc. London*, **123**: 393–409.

PYEFINCH, K. A., 1948. Methods of identification of the larvae of *Balanus balanoides* (L.), *B. crenatus* Brug., and *Verruca stroemia* O. F. Muller. *J. Mar. Biol. Assoc.* **27**: 451–463.

PYEFINCH, K. A., 1949. The larval stages of *Balanus crenatus. Proc. Zool. Soc. London,* **118**: 916–23.

RUNNSTRÖM, S., 1925. Zur Biologie und Entwicklung von *Balanus balanoides* (Linne). Bergen Mus. Aarbok., Naturv. Raekke, Nr. 5.

SANDISON, EYVOR E., 1954. The identification of the nauplii of some South African barnacles with notes on their life histories. *Trans. Roy. Soc. S. Africa,* **34**: 69–101.

YASUGI, R., 1937. On the free-swimming larvae of *Mitella mitella* L. *Botany and Zool., Tokyo,* **V**: 792–796.

SURVIVAL AND GROWTH OF CLAM AND OYSTER LARVAE AT DIFFERENT SALINITIES

H. C. DAVIS

U. S. Fish and Wildlife Service, Milford, Conn.

Adult clams (*Venus mercenaria*) and oysters (*Crassostrea virginica*) are found, both in areas where the salinity is almost oceanic, and in areas where it is low. There is little published information on the effects of salinity on the reproductive processes of the hard clam. Turner and George (1955) reported an experiment in which early larvae of *V. mercenaria* were introduced into the bottom of a glass tube in which layers of sea water of diminishing salinity were placed one above the other. The larvae swam upward, through the sharp gradients that separated the layers, with no loss in velocity until they had passed the boundary between the sea water at 20.0 parts per thousand and that at 15.0 p.p.t. In the latter their velocity decreased and they no longer moved upward. Instead, they swam in a circular pattern just above the interface. Turner, in a personal communication, reported rearing clam larvae to metamorphosis at salinities of 31.0, 28.0, 24.0 and 20.0 p.p.t. He reports, however, that he had a constant mortality in 20.0 p.p.t. until, by the tenth day, he had only about 20 per cent as many living larvae at this salinity as were still living at the higher salinities.

Since Korringa (1941) has reviewed the literature relating to the effects of salinity on several species of oysters, only those works dealing with American oysters will be mentioned here. Ryder (1885), Nelson (1921), Hopkins (1931), Loosanoff (1932) and other investigators have attempted to evaluate, from field data, some of the effects of salinity on various phases of oyster physiology. In addition, Loosanoff (1948, 1952) found experimentally that adult Long Island Sound oysters developed functional spermatozoa and fertilizable eggs at a salinity of 7.5 p.p.t. but that these eggs did not develop normally. In lower salinities gonad development was arrested. He found, however, in one experiment that Long Island Sound oysters, which were already ripe, spawned at salinities as low as 5.0 p.p.t. Butler (1949), in a study of oysters from upper Chesapeake Bay, concluded that gametogenesis was inhibited in 90 per cent of the surviving population until salinity levels rose to about 6.0 p.p.t.

Amemiya (1926) and Clark (1935) studied the salinity range for the development of fertilized eggs, of the American oyster, into shelled larvae. Both concluded that 14.5 or 15.0 p.p.t. was the lower limit for normal development and that 39.0 p.p.t was the upper limit. Amemiya, however, believed the optimum salinity for development was from 25.0 to 29.0 p.p.t., whereas Clark thought the optimum was at 23.0 p.p.t.

Nelson (1921), in New Jersey waters, observed active free-swimming larvae in salinities ranging from 5.17 to 28.80 p.p.t. From this and his observation that the adult oyster closed and refused to feed at salinities below 10.42 p.p.t., Nelson concluded that oyster larvae (p. 38) "may become accustomed to much lower densities than the adult animal will stand, and still remain active."

Prytherch (1934) studied the salinity limits for the attachment and metamorphosis of oyster larvae and found that they could attach in salinities from 5.6 to 32.2 p.p.t., but that beyond these limits (p. 71) "no setting occurred though many of the larvae crawled for periods of over four hours." Moreover, although (p. 71) "setting was accomplished with considerable regularity in salinities ranging from 9.0 to 29.0 p.p.t.," beyond these limits only a small percentage of the larvae was able to complete the process. He believed that the salinity range from 16.0 to 18.0 p.p.t. was optimum for setting.

In the present study we have re-investigated the effect of salinity on development of fertilized eggs of the American oyster, *C. virginica,* into shelled larvae to obtain quantitative data for estimating the relative percentage of eggs developing normally in different salinities. Similar studies were also made on eggs of the hard clam, *V. mercenaria.* In addition, we determined the effects of several lowered salinities on the survival and growth of free-swimming larvae of both clams and oysters after they had reached the straight-hinge stage.

Development of Fertilized Eggs at Different Salinities

Methods

Our laboratory tap water cannot be used, without treatment, to lower the salinity because it contains enough metallic ions, chiefly copper, to be toxic to developing eggs. In Experiment No. 1 we did use tap water to lower the salinity but added a chelator to bind up the excess metal ions. In Experiment No. 2 we used distilled water to dilute our usual sea water. This water was from a Stokes still that discharged into a tin-lined storage tank. In all subsequent experiments a Barnstead BD-2 demineralizer was our source of salt-free water, and this water was stored either in Pyrex carboys or polyethylene tanks.

High salinity water was obtained by evaporation of our sea water in polyethylene containers until the salinity was 44.52 p.p.t. This water was then stored in a Pyrex carboy until used in these experiments. The salinities tested in Experiment No. 3 were obtained by making appropriate dilutions of this high salinity water with our usual sea water (27.0 p.p.t.). In Experiment No. 4 all the salinities were obtained by diluting the high salinity water with demineralized water. The salinities tested in Experiment No. 5 were obtained by diluting our usual sea water with demineralized water.

The animals used in these experiments were spawned in the usual manner (Loosanoff and Davis, 1950; Davis, 1953). Fertilized eggs of both oysters and clams were thus obtained free of the body tissues and excessive sperm that may have affected the results of earlier workers, who used stripped eggs and sperm. For each experiment eggs from several females were pooled and an equal number of eggs was taken from this mixed lot to start cultures at each of the different salinities. All containers were then covered, to prevent loss of water by evaporation, and placed in the constant temperature bath at 23.0° C. Forty-eight hours later the contents of each culture vessel were screened and the number of normal straight-hinge larvae determined. Experimental errors, including transfer of the eggs to the experimental culture vessels, recovery of the larvae and sampling, can probably account for differences of not more than ± 10 per cent in any individual experiment.

Salinity tolerance of developing eggs of V. mercenaria

Eggs of the hard clam of Long Island Sound can develop into normal straight-hinge larvae only within the relatively narrow salinity range of 20.0 to 32.5 p.p.t. (Table I). At a salinity of 35.0 p.p.t. only one per cent or less of the eggs developed into shelled larvae, and at 17.5 p.p.t. none of the eggs developed into normal shelled larvae. Even at 20.0 p.p.t. only 16 to 21 per cent of the eggs developed into straight-hinge larvae and at 32.5 p.p.t. only 34 to 52 per cent reached this stage. Thus, the salinity range for practical work, extending from 22.5 to 30.0 p.p.t., is narrower than the biological or absolute range (20.0 to 32.5 p.p.t.). In our experiments, the optimum salinity for the development of clam eggs was about 26.5 to 27.5 p.p.t.

TABLE I

Comparison of the percentage of eggs, of oysters and clams, that develops to normal straight-hinge larvae in sea water of different salinities. Highest number developing to straight-hinge larvae from each spawning taken as 100 per cent. Experiments No. 3, No. 4 and No. 5 from same spawning

Salinity (in p.p.t.)	Eggs of Long Island Sound oysters		Eggs of Peconic Bay oysters			Eggs of Long Island Sound clams		
	Exp. No. 1	Exp. No. 2	Exp. No. 3	Exp. No. 4	Exp. No. 5	Exp. No. 3	Exp. No. 4	Exp. No. 5
44.5				0				
40.0				0				
35.0			56	29		0	1.0	
32.5			99	35		52	34	
30.0			92	78		54	84	
27.5				75			100	
Control* (26.0–27.0)	89	87	88	88	92	92	92	100
25.0				85			91	
22.5	100	100		94	100		80	84
20.0	90	82		84	76		21	16
17.5	79	91		58	63		0	0
15.0	64	73			48			0
12.5	<0.1	<0.1			18			0
10.0	0	0			0			0
7.5	0	0			0			0
5.0					0			0
2.5					0			0

* Control—Milford Laboratory sea water.

Salinity tolerance of developing eggs of C. virginica

Some eggs of Long Island Sound oysters, conditioned at 26.0 to 27.0 p.p.t., developed into normal straight-hinge larvae in salinities as low as 12.5 p.p.t. (Experiments No. 1 and No. 2, Table I, and Experiment No. 6, Table II). The highest percentage of normal straight-hinge larvae was obtained at a salinity of 22.5 p.p.t. The percentage of normal larvae obtained in salinities below 22.5 p.p.t. decreased progressively, in most experiments, with each successive decrease in salinity down to 15.0 p.p.t. Below 15.0 p.p.t. the percentage of normal larvae decreased abruptly and in a salinity of 12.5 p.p.t. less than 0.1 per cent of the eggs developed normally.

Table II

Comparison of the relative percentage of normal straight-hinge larvae obtained at salinities (a) from eggs of oysters from different areas at the same salinity and (b) from eggs of oysters from the same area at different salinities

	Oysters conditioned and spawned at 26.0–27.0 p.p.t.				Hodges Bar oysters that developed gonads at about 8.74 p.p.t.*		
					Oysters spawned in		
	Long Island Sound Exp. No. 6	Peconic Bay Exp. No. 7	Hodges Bar Exp. No. 6	Hodges Bar Exp. No. 7	7.5 p.p.t. Exp. No. 8	10.0 p.p.t. Exp. No. 8	15.0 p.p.t. Exp. No. 8
Control 26.0–27.0 p.p.t.	100	100	82	91	0	0	0
25.0 p.p.t.	99	86	80	94	0	0	0
22.5 p.p.t.	100	87	90	100	0	0	26
20.0 p.p.t.	100	86	100	91	7	26	72
17.5 p.p.t.	92	76	87	81	50	76	92
15.0 p.p.t.	37	58	60	45	48	100	100
12.5 p.p.t.	<0.1	13	1.4	11	83	92	89
10.0 p.p.t.	0	0	0	0	100	98	78
7.5 p.p.t.	0	0	0	0	99	96	72
5.0 p.p.t.	0	0	0	0	0	0	0
2.5 p.p.t.	0	0	0	0	0	0	0

* These oysters were kept, for four days prior to spawning, at the salinities at which they were induced to spawn.

Eggs of oysters from Peconic Bay, where the salinity may be as high as 31.0 p.p.t., showed about the same percentage developing in each of the lower salinities as did eggs of Long Island Sound oysters, except that a slightly higher percentage of these eggs developed at a salinity of 12.5 p.p.t. (Experiments No. 4 and No. 5, Table I, and Experiment No. 7, Table II).

In salinities above 22.5 p.p.t. the percentage of normal larvae again decreased progressively with each successive increase in salinity up to 35.0 p.p.t. Because of the comparatively high percentage of eggs that developed normally in 35.0 p.p.t., we suspect that at least a few eggs would have developed in 37.5 p.p.t. (not tested), although in 40.0 p.p.t. none developed normally.

The salinity tolerance of eggs of Maryland oysters from Hodges Bar, a low salinity area, differed only slightly from that of eggs of Long Island Sound oysters when both groups of parent oysters were conditioned at 26.0–27.0 p.p.t. (Experiments No. 6 and No. 7, Table II). However, when Maryland oysters from the same area developed gonads in their native habitat, where the salinity was only 8.74 p.p.t. at the time they were collected, and were spawned in salinities of 7.5, 10.0 and 15.0 p.p.t., the eggs developed into normal straight-hinge larvae at 10.0 p.p.t., and larvae, normal in shape and only slightly smaller in size, developed at 7.5 p.p.t. (Experiment No. 8, Table II). Even at 5.0 p.p.t. many of the eggs developed into very early shelled stages before they died. The upper salinity limit for development of normal larvae from eggs of oysters which developed gonads and were spawned at low salinities was also appreciably lower than for eggs of oysters from the same area that developed gonads and were spawned at 26.0–27.0 p.p.t. None of the eggs produced at low salinities developed into normal larvae at salinities above 22.5 p.p.t.

EFFECT OF LOWERED SALINITIES ON GROWTH OF LARVAE

Methods

To determine the effect of lowered salinities on growth of larvae we started with straight-hinge clam and oyster larvae 48 hours old. These larvae were obtained by spawning clams and oysters in sea water at our normal salinity (26.0–27.0 p.p.t.). Several 18-liter cultures of the eggs of each species were then set up in sea water at that salinity and permitted to develop for 48 hours. At the end of this period the larvae from all the cultures were collected on stainless steel screens to give a single combined culture of clam larvae and one of oyster larvae. The number of larvae per ml. in each combined culture was determined and appropriate volumes used to start duplicate cultures of clam larvae and duplicate cultures of oyster larvae at each of the salinities tested.

In both experiments the water in which the larvae were kept was changed every second day. Since the food used was grown in sea water of our normal salinity, it was added before the salinity was adjusted after a change of water. In the first experiment additional food was given to the cultures on the days between changes, as was our usual practice. The salinity could not be adjusted after this additional food was given, however, and it was found that this increased the salinity of the cultures appreciably. The increase in salinity, while only about 0.5 p.p.t. in cultures in which the nominal salinity was 22.5 p.p.t., was as much as 1.5 p.p.t. in cultures nominally at 10.0 p.p.t. and lower.

In the second experiment the salinities were maintained at the nominal level. About 1½ times the usual amount of food was given on the days when the water was changed and the salinity adjusted, but no additional food was given until the next change of water.

Effect on clam larvae

The results of the two experiments on clam larvae were in general agreement in that growth of larvae was comparatively good at salinities of 20.0 p.p.t. and higher (Fig. 1). In both experiments larvae were reared to metamorphosis at these salinities. In the first experiment there was no appreciable difference in growth of the larvae at 20.0 p.p.t., 22.5 p.p.t. and at our normal salinity (26.0–27.0 p.p.t.). However, in the second experiment, in which the salinities were controlled more carefully, the rate of growth of larvae decreased progressively at each successively lower salinity, as shown by the average size at each measuring period. At a salinity of 17.5 p.p.t., although growth of the clam larvae was significantly slower than at normal salinity, some larvae did reach metamorphosis. These larvae were sluggish and, apparently, more susceptible to disease than were larvae kept at higher salinities. Thus, even though some larvae reached metamorphosis, such a high mortality occurred during and immediately after setting that we were unable to follow their growth further.

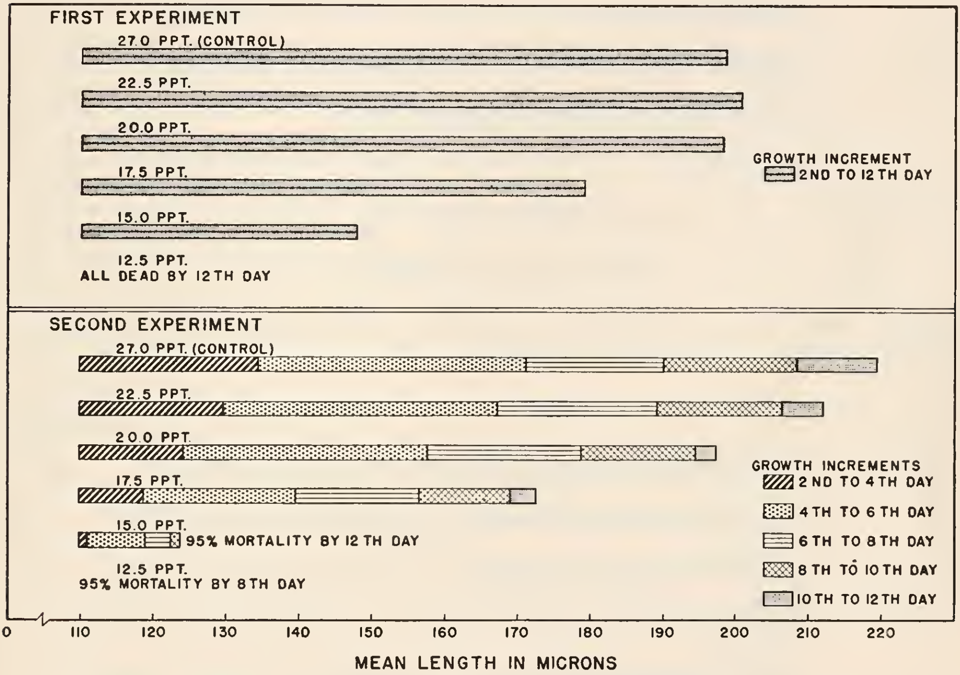


FIGURE 1. Growth of clam larvae at different salinities. Samples were taken and measurements made only at the beginning and termination of the first experiment. In the second experiment, salinities were more carefully controlled and samples, from each of the duplicate cultures at each salinity, were taken every second day. One hundred larvae from each sample were measured.

Clam larvae kept at a salinity of 15.0 p.p.t. grew even more slowly than those kept at 17.5 p.p.t. They were sluggish and susceptible to attack by protozoa, fungus and bacteria. In each experiment some larvae lived more than 12 days, but all died before reaching setting size.

At a salinity of 12.5 p.p.t., a few clam larvae survived for 10 or 12 days, but did not grow. In the second experiment, when samples were taken every two days, a slight but progressive decrease in size of larvae kept at this salinity was noted. In both experiments, it was observed that at salinities of 12.5 p.p.t. and lower, the shells of dead clam larvae were completely disintegrated in approximately 48 hours. The progressive decrease in size of larvae at 12.5 p.p.t., therefore, suggests that the shells, even of living larvae, were being slowly dissolved.

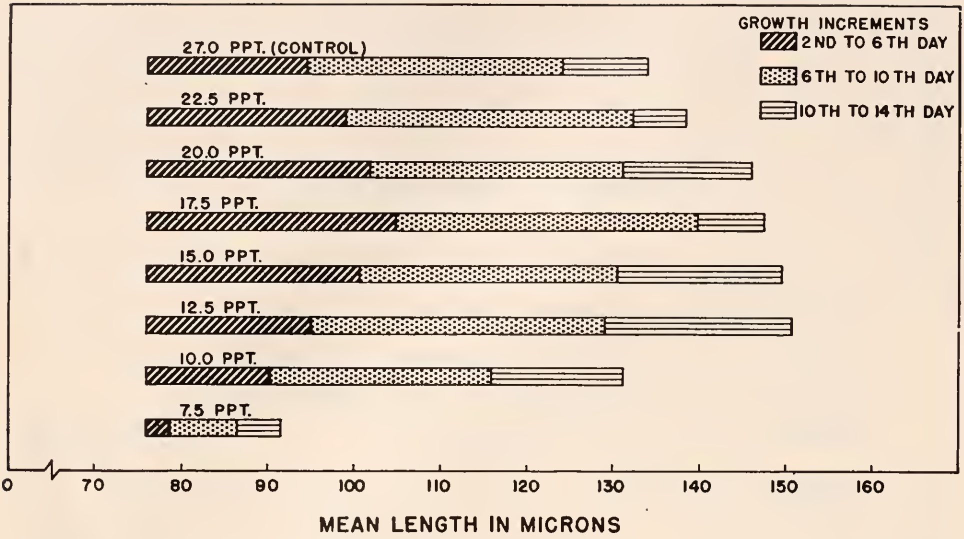


FIGURE 2. Growth of oyster larvae at different salinities. Samples, from each of the duplicate cultures at each salinity, were taken on the sixth, tenth and fourteenth days. One hundred larvae from each sample were measured.

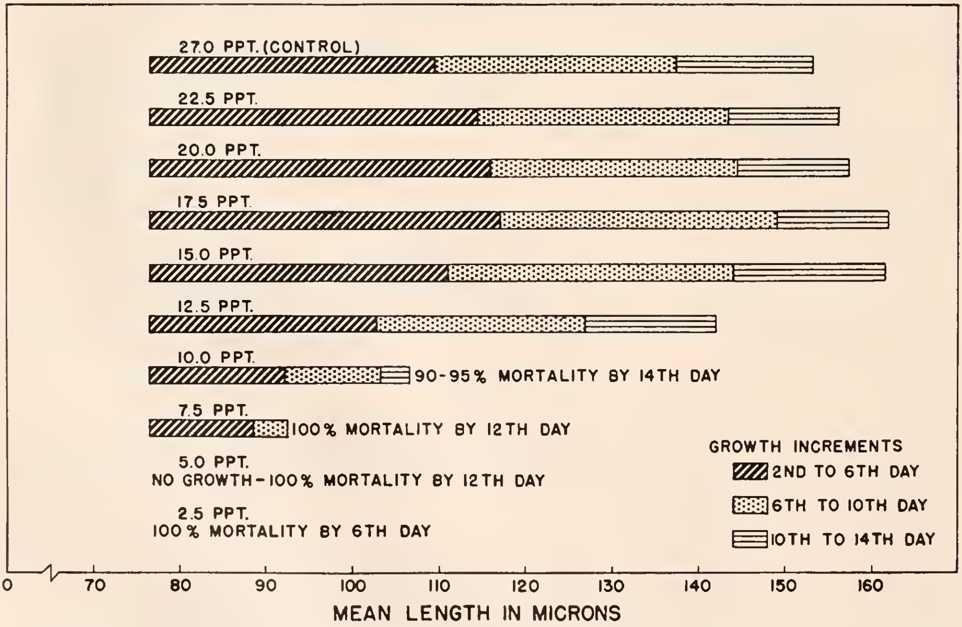


FIGURE 3. Growth of oyster larvae at different salinities. Salinities were more carefully controlled than in the previous experiment. Samples from each of the duplicate cultures at each salinity, were taken on the sixth, tenth and fourteenth days. One hundred larvae from each sample were measured.

At salinities of 10.0 p.p.t. or lower, clam larvae showed no growth and all were dead within six days.

The lower borderline salinity for clam larvae appears to be about 17.5 p.p.t. Clam larvae and set would probably survive and grow slowly at this salinity if all other conditions were nearly ideal, but would probably die if some other environmental factor were unfavorable. It would seem exceedingly doubtful that conditions in nature would ever be so favorable that clam larvae could survive, reach setting stage, and continue to grow at salinities of 15.0 p.p.t. or lower.

Effect on oyster larvae

Oyster larvae grew at a comparatively normal rate in all salinities down to and including 12.5 p.p.t. However, a salinity of 17.5 p.p.t. was optimum, by a slight margin, for growth of larvae at least through the tenth day (Figs. 2 and 3). In both experiments larvae kept in a salinity of 15.0 p.p.t. had, by the fourteenth day, attained an average length equal to or slightly greater than those kept at 17.5 p.p.t. In the first experiment the larvae kept at 12.5 p.p.t. were, by the fourteenth day, slightly larger than those at any other salinity, and even the larvae kept at 10.0 p.p.t. were almost as large as the controls (Fig. 2). In the first experiment, however, due to the addition of food on the days when the water was not changed, the salinities of these cultures actually ranged from 12.5 to about 14.0 p.p.t. and from 10.0 to 11.5 p.p.t., respectively. In the second and other experiments, in which the salinity was more carefully controlled, growth of larvae at 12.5 p.p.t. was appreciably slower than at 15.0 p.p.t., while larvae kept at 10.0 p.p.t. grew considerably slower than the controls, and in some experiments mortality was high.

In the first experiment, in which the salinity of the cultures nominally at 7.5 p.p.t. actually ranged from 7.5 to 9.0 p.p.t., some oyster larvae lived through the 14 days of the experiment, although growth was slow and mortality high (Fig. 2). In the second experiment, and others in which the salinity was held at 7.5 p.p.t., the larvae appeared to feed and seemed quite normal for the first few days, even though growth was very slow (Fig. 3). By the eighth to tenth day at this salinity, however, oyster larvae appeared moribund and by the twelfth day mortality was almost complete.

At a salinity of 5.0 p.p.t. oyster larvae appeared moribund within 48 hours. After four days almost all were completely dead, and in only a few could ciliary motion be detected.

Thus far, we have the results of only one experiment using larvae of oysters from low salinity areas in Maryland. While the results of a single experiment are not always reliable, this experiment indicated that when these oysters are conditioned at a salinity of 26.0–27.0 p.p.t. their larvae do not tolerate any lower salinities than do larvae from Long Island Sound oysters conditioned at the same salinity. As a matter of fact, in this experiment the optimum growth of the Maryland larvae was at 22.5 p.p.t., and the rate of growth decreased progressively with each successive decrease in salinity below this optimum.

Effect on older oyster larvae

The results of the above experiments, in which we started with straight-hinge larvae, could be interpreted as indicating that larger larvae (10 to 14 days old) can grow as fast as or faster, at a salinity of 15.0 p.p.t. (or even 12.5 p.p.t. in the first experiment), than at 17.5 p.p.t. We believed, however, that this was because the larvae at 17.5 p.p.t., being larger at 10 days, were more severely handicapped by insufficient food. Davis and Guillard (in press) have shown that it requires approximately four times the quantity of food given these cultures to maintain maximal growth of larvae after they reach an average length of 140 μ, yet such a quantity of food would have handicapped the smaller or slower growing larvae at the other experimental salinities.

To test the above hypothesis in another experiment we determined the rate of growth of older larvae at salinities of 26.0–27.0 p.p.t. (control), 17.5, 15.0, 12.5, 10.0 and 7.5 p.p.t. A culture of oyster larvae, that had been reared to a mean length of 165 μ at normal salinity, was divided into six smaller cultures and one of these was kept at each of the above salinities. Eight days later it was found that the larvae kept at 17.5 p.p.t. had grown the fastest, while there was very little difference in size between the controls and those kept at 15.0 or 12.5 p.p.t. (Table III). The larvae kept at 10.0 p.p.t., however, had increased in size only about one-half as much as the controls, and those kept at 7.5 p.p.t. had increased only about one-fourth as much as the controls.

TABLE III

Comparison of growth of older oyster larvae at different salinities. Measurements are in microns

	Control 26.0–27.0 p.p.t.	17.5 p.p.t.	15.0 p.p.t.	12.5 p.p.t.	10.0 p.p.t.	7.5 p.p.t.
Initial mean length	165.55	165.55	165.55	165.55	165.55	165.55
Length after 8 days at different salinities	206.30	214.00	203.05	205.75	186.80	175.49
Increase	40.75	48.45	37.50	40.20	21.25	9.94

These cultures were continued for several more days to get an indication of the setting rate of larvae in the different salinities. Those kept in 17.5 p.p.t. gave significantly more spat than any of the other cultures, but setting was also good at 15.0 and 12.5 p.p.t. A few spat were obtained at 10.0 p.p.t. but the larvae kept at 7.5 p.p.t. all died before metamorphosis.

DISCUSSION

Our experiments on the development of fertilized clam eggs indicate that even with a salinity as high as 22.5 p.p.t., the reproductive potential of clams may be reduced as much as 15 to 20 per cent. If the salinity is reduced to 20.0 p.p.t., the reproductive potential of clams is reduced 80 to 85 per cent and if the salinity over the spawning beds should be as low as 17.5 p.p.t., there appears to be no possibility of obtaining normal larvae.

Once clam larvae have attained the straight-hinge stage, we find, as did Turner (personal communication), that the larvae grow quite well at 20.0 p.p.t. but contrary to Turner's results we find no significant mortality at this salinity. Turner and George's (1955) observation that the larvae swam upward, the normal reaction, until they came into the sea water at 15.0 p.p.t. also appears significant. We find that at both 17.5 and 15.0 p.p.t. the larvae appear sluggish, grow slowly and suffer high mortality either prior to reaching setting stage (15.0 p.p.t.) or during metamorphosis (17.5 p.p.t.).

The minimum salinity at which a good percentage of clam eggs develops into straight-hinge larvae is 22.5 p.p.t. Once the larvae have attained this stage, however, they survive and grow well at a salinity as low as 20.0 p.p.t. Thus, as Loosanoff, Miller and Smith (1951) showed for the temperature requirements of eggs and larvae of *V. mercenaria,* the embryonic stages cannot tolerate as wide a range of salinities as can the larval stages. Similarly, Chanley (in press) found that small juvenile clams (1.8 to 3.6 mm. in length) survived for a month or more at 15.0 p.p.t. but died in salinities of 12.5 p.p.t. or lower, while larger juveniles (5.0 to 21.5 mm. in length) survived at 12.5 p.p.t.

Much additional research is needed to find the minimum salinities at which adult clams develop gonads, to find whether the salinity at which the parents develop gonads influences the salinity tolerance of the eggs and larvae, and to find whether races tolerant of low salinities exist or can be developed.

By comparison with the status of research on clams, the relation of salinity to the reproductive processes of oysters appears to be fairly well documented. Thus, the findings of Loosanoff (1948, 1952) and Butler (1949) appear to agree quite well that 6.0 to 7.5 p.p.t. is the minimum salinity for the development of gametes by the American oyster. Additional research is required, however, to determine more clearly the value of gametes produced at low salinities.

In general, our results on the development of fertilized eggs of oysters conditioned at a salinity of 27.0 p.p.t. are in close agreement with those of Amemiya (1926) and Clark (1935). However, possibly because of improvements in technique, use of larger cultures and repeated trials, or due to differences in the salinities at which the oysters developed gonads, we have been able to demonstrate that a few eggs of such oysters will develop normally at 12.5 p.p.t. or about 2.5 p.p.t. lower than found by earlier workers.

The very close agreement of the results of Amemiya (1926), Clark (1935), and ours with oysters conditioned at 26.0–27.0 p.p.t., researches so widely separated in time, space and populations sampled, suggested that the salinity tolerance of eggs of the American oyster was quite similar throughout the range of the mollusk. Preliminary tests with Maryland oysters from Hodges Bar, a low salinity area, that had been artificially conditioned at 27.0 p.p.t., appeared to confirm this suggestion. Additional research, however, showed that this was not true when these oysters developed gonads in their native habitat where the salinity was only 8.74 p.p.t. at the time the oysters were collected. When these oysters were spawned at salinities of 7.5, 10.0 or 15.0 p.p.t., comparatively normal larvae were obtained in a salinity as low as 7.5 p.p.t. It may be that eggs of oysters developing gonads at even lower salinities can develop at salinities below 7.5 p.p.t.

Additional research is needed to determine the value of larvae developing at such low salinities. Will such larvae survive, grow and set? Will they grow and set at lower salinities than larvae developing at higher salinities?

Our observation that, once they have reached the straight-hinge stage, oyster larvae may live for some time at salinities as low as 5.0 p.p.t. suggests that the larvae Nelson (1921) reported swimming at a salinity of 5.17 p.p.t., and those observed by many other workers at low salinities, may simply be survivors of larval populations accidentally carried into these low salinities. Our observations, even of older larvae, at 10.0 p.p.t., indicate that growth at this salinity is extremely slow although a very few of the larvae kept at this salinity did set. Perhaps larvae from oysters that develop gonads at low salinities survive better and grow faster at salinities of 10.0 p.p.t. and lower than do larvae from oysters conditioned at 26.0–27.0 p.p.t. Otherwise, our observations on growth, coupled with Prytherch's (1934) observations on the setting of larvae at different salinities, would seem to suggest that oyster sets, occurring in areas where the salinity is only 10.0 p.p.t. or lower, are dependent upon larvae that grow almost to setting size at higher salinities and are carried to low salinity setting areas as practically fully mature larvae. Moreover, Chanley's results (in press) indicate that recently set spat, like the larvae, grow best at salinities near 17.5 p.p.t. and that growth is significantly retarded by salinities of 10.0 p.p.t. or lower. Unlike the larvae some of his spat grew slowly at a salinity of 5.0 p.p.t. although only about 40 per cent survived at this salinity.

The author wishes to express his deep appreciation of the valuable counsel and assistance given by Dr. V. L. Loosanoff, Director of Milford Laboratory. Many thanks are also due to Mr. J. B. Glancy for the Peconic Bay oysters, to our colleagues at the Annapolis laboratory for the Maryland oysters, to Mr. C. A. Nomejko for preparing the figures and slides, to Miss Norma Pritchard and Miss Beverly Boyne for many of the larval measurements, and to Miss Rita Riccio for her careful editing of the manuscript.

SUMMARY

1. The optimum salinity for the development of straight-hinge larvae from eggs of clams from Long Island Sound is about 27.5 p.p.t.

2. The salinity range for development of eggs of these clams is from 20.0 p.p.t., at which salinity only 16 per cent to 21 per cent of the eggs develop, to 35.0 p.p.t., at which salinity only 1 per cent or less of the eggs develops normally.

3. The optimum salinity for growth of clam larvae after they reach the straight-hinge stage is 27.5 p.p.t. or higher, while 15.0 p.p.t. is the lowest salinity at which appreciable growth occurs, and 17.5 p.p.t. is the lowest at which we were successful in rearing clam larvae to metamorphosis.

4. Both the optimum salinity and the salinity range for the development of straight-hinge larvae from eggs of the American oyster appear to be governed by the salinity at which the parent oysters develop gonads.

5. The optimum salinity for the development of eggs of oysters from Long Island Sound, Peconic Bay, and Hodges Bar, Maryland was about 22.5 p.p.t. when these oysters developed gonads at a salinity of 26.0–27.0 p.p.t.

6. When Hodges Bar oysters developed gonads at a salinity of approximately 8.74 p.p.t. the optimum salinity for the development of their eggs was between

10.0 and 15.0 p.p.t. and appeared to be dependent upon the salinity at which the parent oysters were kept immediately prior to spawning.

7. The salinity range for development of normal straight-hinge larvae from eggs of these low salinity oysters was from 7.5 to 22.5 p.p.t., whereas the range for eggs from oysters conditioned at 26.0–27.0 p.p.t. was from 12.5 to above 35.0 p.p.t.

8. The optimum salinity for growth of larvae of Long Island Sound oysters, conditioned and spawned at 26.0–27.0 p.p.t., was 17.5 p.p.t.

9. The optimum salinity for growth of larvae of Hodges Bar oysters, conditioned and spawned at 26.0–27.0 p.p.t., appeared to be about 22.5 p.p.t.

10. It is still undetermined whether the optimum salinity for growth of larvae is influenced by the salinity at which the parent oysters develop gonads.

LITERATURE CITED

Amemiya, I., 1926. Notes on experiments on the early developmental stages of the Portuguese, American and English native oysters, with special reference to the effect of varying salinity. *J. Mar. Biol. Assoc.*, **14**: 61–175.

Butler, P. A., 1949. Gametogenesis in the oyster under conditions of depressed salinity. *Biol. Bull.*, **96**: 263–269.

Chanley, P. E., 1957. Survival of some juvenile bivalves in water of low salinity. *Proc. Nat. Shellfish. Assoc.*, Vol. 48 (in press).

Clark, A. E., 1935. Effects of temperature and salinity on early development of the oyster. *Prog. Rept., Atl. Biol. St., St. Andrews N. B., No. 16:* 10.

Davis, H. C., 1953. On food and feeding of larvae of the American oyster, *C. virginica. Biol. Bull.*, **104**: 334–350.

Davis, H. C., and R. R. Guillard. Relative value of ten genera of micro-organisms as foods for clam and oyster larvae. *U. S. Dept. of Interior, Fish and Wildlife Service* (in press).

Hopkins, A. E., 1931. Factors influencing the spawning and setting of oysters in Galveston Bay, Texas. *Bull. Bur. Fisheries*, **47**: 57–83.

Korringa, P., 1941. Experiments and observations on swarming, pelagic life and setting of the European flat oyster, *Ostrea edulis* L. *Arch. Néer. Zool.*, **5**: 1–249.

Loosanoff, V. L., 1932. Observations on propagation of oysters in James and Corrotoman Rivers and the Seaside of Virginia. *Va. Comm. of Fish., Newport News, Va.*, 1–46.

Loosanoff, V. L., 1948. Gonad development and spawning of oysters (*O. virginica*) in low salinities. *Anat. Rec.*, **101**: 55.

Loosanoff, V. L., 1952. Behavior of oysters in water of low salinities. *Conv. Address, Nat. Shellfish. Assoc.*, 135–151.

Loosanoff, V. L., and H. C. Davis, 1950. Conditioning *V. mercenaria* for spawning in winter and breeding its larvae in the laboratory. *Biol. Bull.*, **98**: 60–65.

Loosanoff, V. L., W. S. Miller and P. B. Smith, 1951. Growth and setting of larvae of *Venus mercenaria* in relation to temperature. *J. Mar. Res.*, **10**: 59–81.

Nelson, T. C., 1921. Aids to successful oyster culture. I. Procuring the seed. *N. J. Agric. Exp. Sta., Bull. 351,* 1–59.

Prytherch, H. F., 1934. The role of copper in the setting, metamorphosis and distribution of the American oyster, *Ostrea virginica. Ecol. Monogr.*, **4**: 49–107.

Ryder, John A., 1885. New system of oyster culture. *Science*, **6**: 465–467.

Turner, H. J., and C. J. George, 1955. Some aspects of the behavior of the quahaug, *Venus mercenaria*, during the early stages. *Eighth Rept. on Invests. of Shellfish. of Mass., Dept. of Nat. Res.*, 5–14.

RESPIRATION IN TISSUES OF GOLDFISH ADAPTED TO HIGH AND LOW TEMPERATURES

DONALD R. EKBERG

Department of Physiology, University of Illinois, Urbana, Illinois

Poikilothermic animals adapted to low temperatures are very different physiologically from warm-adapted animals. These differences have been detected in terms of lethal temperatures, metabolic variations, and other gross changes. In recent years an interest has arisen in the cellular mechanisms of these changes.

Peiss and Field (1950) measured the oxygen consumption of minced brains and sliced livers from cold- (0°–1° C.) adapted cod (*Boreogadus saida*) and from warm- (25° C.) adapted golden orfe (*Idus melanotus*). Differences in Q_{O_2} of both liver and brain were in the same direction as for intact animals. Liver and brain excised from the cold-adapted animals consumed oxygen at a higher rate than did the same tissues excised from the warm-adapted animals when measured at the same temperature. The Q_{10} of the orfe tissues was greater than that of the cod tissues, particularly in the 0° C.–10° C. temperature range.

Similar results were reported for goldfish (*Carassius auratus*) brain breis by Freeman (1950). However, for muscle tissue, the oxygen consumption was greater in muscle from warm-adapted than from cold-adapted fish. These findings on muscle were corroborated by the studies of Suhrman (1955).

In an attempt to localize the cellular mechanisms of temperature adaptation, Precht and his co-workers have recently shown alterations in enzyme activity in tissues from animals adapted to various temperatures (Precht, Christophersen and Hensel, 1955). Christophersen and Precht (1952) observed that the catalase activity of the liver of the carp (*Carrasius vulgaris* Nils) adapted to 1° C. was greater than that of liver from fish adapted to 26° C. However, the dehydrogenase activity (rate of decolorization of methylene blue) was found to be greater in the liver of the warm-adapted animals. On the other hand, Precht (1951) found that the liver catalase activity of the eel (*Anguilla vulgaris* L.) adapted to 26° C. was greater than that of liver catalase from animals adapted to 11° C.; the dehydrogenase activity of liver or muscle brei decreased as the adaptation temperature increased; the catalase activity of eel muscle appeared to be independent of the adaptation temperature.

In potato beetles (*Leptinotarsa decemlineata* Say) adapted to 3° C. and 24° C. Precht (1953) found that blood catalase and tissue succinic dehydrogenase were greater in their activity in the cold-adapted than in the warm-adapted insects. The high temperature of adaptation was only 17.5° C.; Precht also observed that higher temperatures (24° C.) greatly increased locomotor activity in these insects with a concomitant increase in dehydrogenase activity.

MATERIALS AND METHODS

Adaptation of animals. The experimental animals (*Carassius auratus*) were obtained from Grassyfork, Inc., Martinsville, Indiana. These goldfish varied in weight from 20 to 80 grams. Upon arrival at the laboratory they were put into a series of tanks which were continually being renewed with aerated dechlorinated water. Pulverized Purina Dog Chow was placed into these tanks for one day. After this period the fish were transferred to continuously aerated water in the adaptation tanks, which consisted of seven-gallon aquaria immersed in water at 10° and 30° C. The fish were kept in these adaptation tanks for two weeks without feeding prior to experimentation. The water in the tanks was completely changed every week.

Preparation of tissues for manometric studies. Following the adaptation period a fish was removed from the adaptation tank, blotted with a paper towel, and weighed. The animals were then killed by severing the spinal cord immediately posterior to the head with a pair of scissors. The brain was removed first and blotted to remove blood and meninges; then the body cavity was opened by two connecting incisions—one median ventral and one ventral transverse. Thus, the liver could be removed piece-wise by scraping it away from the intestine, spleen, and gall bladder. Then the gill filaments were cut away from the gill arches, washed in frog Ringer's, and placed in the medium described below. Gills are quite thin and thus may be used in a Warburg vessel without homogenization or mincing.

Ringer's Solution

NaCl	6.5 g./l.	70 parts
KCl	0.15 g./l.	
$CaCl_2$	0.12 g./l.	
$NaHCO_3$	0.20 g./l.	
Na_2HPO_4	0.01 g./l.	
0.3 *M* glucose		5 parts
0.16 *M* Na-pyruvate		4 parts
0.1 *M* Na-succinate		7 parts
0.16 *M* Na-L glutamate		4 parts
Phosphate buffer	pH 7.5	
13 parts	0.021 *M* Na_2HPO_4	
1 part	0.160 *M* KH_2PO_4	

The above mixture was kept frozen in the refrigerator prior to use. New mixtures were made up weekly. The medium is similar to that prepared by Krebs (1950) for mammalian tissues. Each vessel contained 2.5 ml. of the above medium and gill filaments plus 0.2 ml. of 10 per cent KOH and fluted Whatman No. 1 filter paper in the center well.

Brain and liver homogenates did not survive well at temperatures above 20° C. Thus, if the oxygen consumption were measured at temperatures of 10°, 18°, and 26° C. successively with the same tissues, it was observed that the oxygen consumption at 26° C. was decidedly lower than if the Q_{O_2} had been measured initially at this temperature. The homogenate technique was discarded, therefore, in favor of the following: The tisues were removed from the animal and placed on filter paper on a Petri dish over ice. The tissues were then minced with a razor blade and placed into the previously mentioned medium. It was found that the oxygen consumption of a given sample of tissue could be measured at two temperatures (14° and 22° C., 10° and 30° C.) without appreciable loss of activity at the higher temperature.

Cyanide inhibition of respiration. The method used in these studies was essentially that of Robbie (1948). The center well contained 0.4 ml. of 0.42 M $Ca(CN)_2$ in 10 per cent $Ca(OH)_2$. This mixture equilibrates with the vessel fluid to give a final cyanide concentration of 10^{-3} M. However, the equilibration time is about one hour at 26° C. and to alleviate this delay 0.1 ml. of 2.8×10^{-2} M KCN was placed into the vessel fluid of 2.7 ml., thus giving a final concentration of 10^{-3} M cyanide. Samples of the vessel fluid were removed at the end of an experiment and analyzed for cyanide by the phenolphthalin method of Robbie (1948). It was found that the equilibrium concentration of cyanide was not appreciably altered by the above procedure. The control vessels contained 0.4 ml. of 10 per cent $Ca(OH)_2$.

Iodoacetate inhibition of respiration. Since iodoacetate produces its effect on living systems by combining with SH groups of various enzymes, it is to be expected that its action will be non-specific. However, Adler and co-workers (1938) have shown that triose phosphate dehydrogenase (TDH) is more sensitive to iodoacetate than a number of other sulfhydryl enzymes. Recently Kelly and co-workers (1955) have used iodoacetate in small concentration (5.4×10^{-4} M) to block glycolysis in adrenal tissue Thus, it was hoped that a similar study utilizing goldfish gills would yield information concerning the activity of the hexose monophosphate shunt in this tissue.

The following medium was used:

Control		*Experimental*	
0.3 M glucose	5 parts	0.3 M glucose	5 parts
Phosphate buffer	30 parts	Phosphate buffer	30 parts
Ringer's solution	70 parts	15 mg. iodoacetic acid in 100 ml. Ringer's solution	70 parts

Thus, the final concentration of iodoacetate in the vessel was 5.4×10^{-4} M.

It was found that iodoacetate produced only a small inhibitory effect on the oxygen consumption of whole gills. Before considering that this lack of marked inhibition is due to a high level of activity of the hexose monophosphate shunt it was necessary to know whether the iodoacetate was entering the cells. At pH 7.5 the iodoacetic acid is highly ionized, and if one assumes that only the un-ionized form enters the cells, then a method for increasing the amount of the un-ionized form would be to lower the pH. However, a drop of 2–3 pH units would decidedly inhibit oxygen consumption; thus the homogenate technique was employed to facilitate diffusion of the iodoacetic acid into the cells.

The results of the rate-temperature studies for goldfish brain, liver, and gills are given in tabular form in Tables I and II and in graphic form in Figure 1. The data for the cyanide and iodoacetate inhibition studies are given in Table III. Two tests were used for the level of significance: Student's Test as modified by Fisher and reported by Patau (1943) and the Mann-Whitney U Test as reported by Siegel (1956). The latter test is much simpler to run than the t test, and since it is a nonparametric test as opposed to the parametric t test, one need not concern

TABLE I

Oxygen consumption of brain and liver from goldfish adapted to 10° C. and from goldfish adapted to 30° C.

Temperature of measurement in ° C.	Q_{O_2} (μl./mg. dry weight/hour)				P value	
	Adaptation temperatures					
	10° C.	Number of animals	30° C.	Number of animals	*t*	M-W
Brain						
10	0.813	5	0.700	5	0.37	0.11
14	0.992	5	0.887	5	0.90	0.42
22	1.53	5	1.50	4	0.93	0.45
30	2.27	4	2.18	4	0.92	0.17
Liver						
10	0.621	4	0.454	4	0.76	0.10
14	0.907	6	0.649	5	0.04	0.09
22	1.39	3	0.849	3	0.08	0.05
26	1.36	8	1.19	6	—	0.286
30	1.44	4	1.41	4	—	0.56

himself with the stringent assumptions of the *t* test. The probabilities for assuming random distribution are given for both tests in Tables I–III. The five per cent level is taken as the minimum level of significance.

Brain and liver oxygen consumption. The differences in oxygen consumption for brain and for liver from goldfish adapted to 10° C. and 30° C. as shown in Table I and Figure 1 are small and not statistically significant.

Gill oxygen consumption. The oxygen consumption of gills from goldfish adapted to 10° C. was in all cases greater than that of gills from fish adapted to 30° C. as shown in Table II and Figure 1c. However, in the sequence of meas-

TABLE II

Oxygen consumption of gills from goldfish adapted to 10° C. and from goldfish adapted to 30° C.

Temperature of measurement in ° C.	Month of study	Q_{O_2} (μl./mg. dry weight/hour)				P value	
		Adaptation temperature					
		10° C.	Number of animals	30° C.	Number of animals	*t*	M-W
10	August	0.405	3	0.255	3	0.07	0.002
14	July	0.706	3	0.448	3	0.14	0.10
22	July	1.37	4	0.858	4	0.001	0.014
30	August	1.60	3	1.00	3	0.013	0.05
10	February	0.257	8	0.106	6	0.002	0.002
18	February	0.557	8	0.262	6	0.0006	0.001
26	February	1.074	8	0.522	4	0.0002	0.002

urements from July to February all of the curves (Fig. 1c) are shifted down on the rate axis. This indicates a seasonal variation upon which temperature acclimation is superimposed. Homogenized gills (controls for the IAA experiments) had a lower oxygen consumption, 0.553 μl/mg./hr. for the 10° C. gills and 0.193 μl/mg./hr. for the 30° C. gills than did whole gills (Table II), but the Q_{O_2}

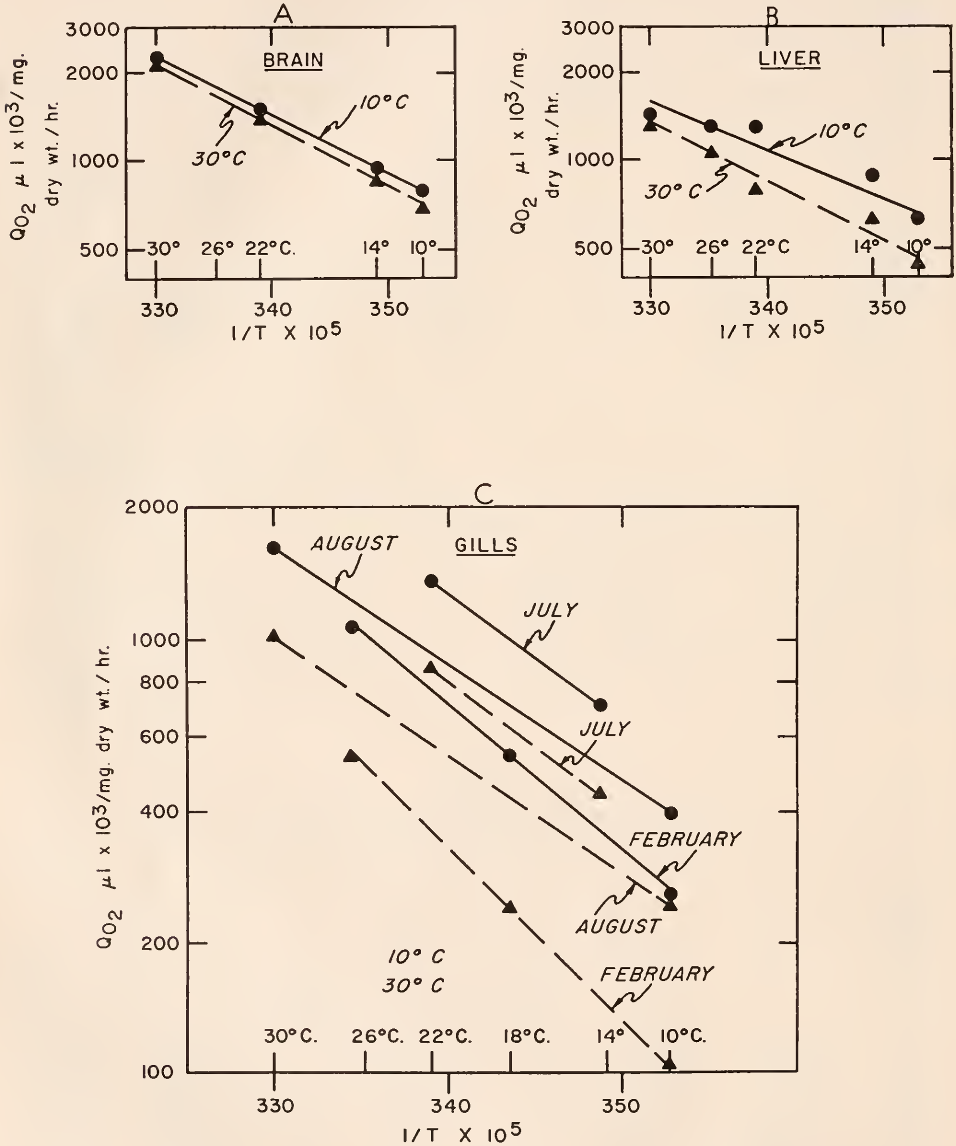


FIGURE 1. Arrhenius plot of the oxygen consumption of brain (a), liver (b), and gills (c) from goldfish adapted to 10° C. (solid lines) and from goldfish adapted to 30° C. (broken lines). See Tables I and II for statistical data.

for gill homogenates from cold-adapted fish was significantly higher than homogenates from warm-adapted fish.

Cyanide inhibition of oxygen consumption. The results of the effects of 10^{-3} *M* cyanide on the oxygen consumption of goldfish liver and gills are summarized in Table III. A small non-significant difference is noted for liver, while the gills from fish adapted to 30° C. were more resistant to cyanide poisoning than gills from fish adapted to 10° C.

Iodoacetate inhibition of oxygen consumption. The data for this group of experiments are summarized in Table II. Iodoacetate had little or no effect on the oxygen consumption of intact gills, yet IAA strongly inhibited the gill homogenates. This suggests that permeability is the limiting factor. This is in contrast to the cyanide studies in which the effect of cyanide was fairly rapid (inhibition was maximum after a 10-minute equilibration period). Since no definite statements can be made concerning the number of cells broken in the homogenate or of the amount of iodoacetate that actually entered the cells, it is impossible to estimate accurately the amount of metabolic activity accounted for by the hexose monophosphate shunt. However, these results do indicate that gill homogenates from 10° C.-adapted fish are more resistant to iodoacetate poisoning than are gills from fish adapted to 30° C.; this is opposite to the effect of cyanide.

TABLE III

The effect of 10^{-3} M cyanide (Nov.–Dec.–Jan.) and 5.4×10^{-4} M iodoacetic acid (Feb.–March) on the oxygen consumption of tissues from goldfish adapted to 10° C. and from goldfish adapted to 30° C. when measured at 26° C.

Tissues	Per cent inhibition of oxygen consumption				P value	
	Adaptation temperature					
	10° C.	Number of animals	30° C.	Number of animals	t	M-W
Cyanide						
Liver	85.9	7	81.2	7	0.17	0.13
Gills (whole)	79.1	7	57.5	7	0.024	0.009
Iodoacetate						
Gills (homogenized)	52.6	7	77.4	7	—	0.027

DISCUSSION

In view of the negligible temperature adaptation differences in oxygen consumption observed in goldfish liver and brain, it appears that further studies of oxygen consumption in these tissues would be of doubtful value. On the other hand goldfish gills appear to be an ideal tissue for this type of study in that gills from fish adapted to 10° C. do show a significantly higher rate of oxygen consumption than do gills from fish adapted to 30° C. when measured at temperatures between 10° C. and 30° C.

Figure 2 indicates that there is a definite decrease in oxygen consumption by goldfish gills during the period from July to February regardless of the adaptation temperature. Seasonal variations in oxygen consumption of intact goldfish have not been reported. However, Hoar (1955, 1956) has demonstrated a greater resistance to high temperature in summer than in winter for goldfish adapted to the same temperature.

A comparison of the rate-temperature curves reveals no apparent difference between slopes of oxygen consumption by gills from animals adapted to 10° C. or 30°.C. for any given month of measurement. Yet, this does not exclude the possibility that an enzyme or a group of enzymes may change in such a way that the net effect is unnoticeable.

Precht *et al.* (1955), as previously mentioned, have shown that enzymes such as dehydrogenases and catalase change their activity with a change in adaptation temperature. However, he has worked with homogenates and thus may not conclude that a certain enzyme has increased or decreased in concentration, but only that its *activity* has increased or decreased. One must consider the following possibilities as affecting the activity of a certain enzyme:

(1) change in permeability of the cell membrane to substrate molecules;
(2) release within the cell of some activating agent or of some inhibitor;
(3) inactivation of a chemical inhibitor or activating agent initially present within the cell;
(4) increase or decrease of the actual amount of enzyme present;
(5) change in intrinsic properties of the enzyme itself.

Present data do not allow a distinction to be made among these possibilities. However, Kaplan (1954, 1955), Fraser and Kaplan (1955), and Kaplan and Paik (1956) have attempted to describe the Euler effect (an increase in enzyme activity when the enzyme is extracted from the cell) on yeast catalase by progressive elimination of (1) to (4) above, thus leaving (5) as the operating mechanism.

Cyanide inhibition of gill oxygen consumption. The increased resistance of gills from 30° C.-adapted goldfish to 10^{-3} M cyanide, as measured by oxygen consumption, may be interpreted in a number of ways.

(1) There is an increase in the amount of one or more of the cytochromes in the gills from 30° C.-adapted fish.
(2) The cytochrome system of the gills from 30° C.-adapted fish is changed in such a manner that it is less sensitive to cyanide.
(3) An oxidative pathway which is less sensitive to cyanide than is the cytochrome system is increased in activity in the gills from 30° C.-adapted fish.

In view of the fact that the oxygen consumption of the gills from 30° C.-adapted fish is lower than that of gills from 10° C.-adapted fish, it does not appear that (1) offers an adequate explanation of the observed differences in cyanide sensitivity. The second possibility (2) must be considered as a possible explanation and merits further study. It seems more probable, however, that in the gills of fish adapted to high temperatures the oxidative pathway is shifted away from the cytochrome system (3). If the activity of the oxidases and aerobic dehydrogenases were increased, this in turn could increase the activity of the peroxidases and catalase.

Iodoacetate inhibition of gill oxygen consumption. If one may assume that iodoacetic acid is primarily blocking triose phosphate dehydrogenase, then it would appear that the hexose monophosphate shunt is operating at a higher capacity in the gills of 10° C.-adapted fish. Such an increase in shunt activity suggests (Glock and McClean, 1954) a correlation between protein synthesis and the shunt. In cold-adapted fish gills there may exist a higher rate of protein synthesis than in warm-adapted fish gills. This in turn would increase the need of cold-adapted fish gills for oxygen.

These inhibitor studies suggest that the cytochrome system has become less sensitive to cyanide poisoning or that an alternate pathway less sensitive to cyanide than the cytochrome system has increased in activity in gills from fish adapted to 30° C., and that the hexose monophosphate shunt may be more active in gills from 10° C.-adapted fish.

The writer is deeply indebted to Professor C. Ladd Prosser for his helpful suggestions during the experimentation period and during the preparation of the manuscript.

Summary and Conclusions

1. No statistical differences were observed in the oxygen consumption by either brain or liver homogenates from goldfish adapted to 10° C. and from fish adapted to 30° C., when measurements were at temperatures between 10° and 30° C.
2. The oxygen consumption of gills from goldfish adapted to 10° C. was found to be significantly higher at all temperatures of measurement, than the oxygen consumption of gills from goldfish adapted to 30° C.
3. No difference was noted in the slopes of the rate-temperature curves of the gills from fish adapted to 10° and from fish adapted to 30° C. as measured by oxygen consumption.
4. A seasonal variation in the oxygen consumption of goldfish gills was noted, in that the oxygen consumption of the gills decreased from July to February. This was true regardless of the temperature of adaptation. However, significant differences were observed between 10° C. and 30° C. gills throughout the experimental period.
5. The oxygen consumption of gills from fish adapted to 30° C. was inhibited to a lesser extent by 10^{-3} *M* cyanide than was the oxygen consumption of gills from fish adapted to 10° C.
6. The oxygen consumption of gills from goldfish adapted to 10° C. was inhibited to a lesser extent by 5.4×10^{-4} *M* iodoacetate than was the oxygen consumption of gills from fish adapted to 30° C.
7. It is suggested that temperature acclimation may be associated with changes in relative activities of CN^- and IAA sensitive enzymatic pathways.

LITERATURE CITED

Adler, E., H. von Euler and G. Gunther, 1938. Dehydrasen und Jodessigsäure. *Skand. Arch. Physiol.*, **80**: 1–15.

Christophersen, J., and H. Precht, 1952. Untersuchungen zum Problem der Hitzeresistenz I. Versuche an Karauschen (*Carassius vulgaris* Nils). *Biol. Zentralbl.*, **7**: 313–326.

Frazer, M. J., and J. C. Kaplan, 1955. The alteration of intracellular enzymes. III. The effect of temperature on the kinetics of altered and unaltered yeast catalase. *J. Gen. Physiol.*, **38**: 515–547.

Freeman, J. A., 1950. Oxygen consumption, brain metabolism and respiratory movements of goldfish during temperature acclimatizations; with special reference to lowered temperatures. *Biol. Bull.,* **99**: 416–424.

Glock, G. E., and P. McClean, 1954. Levels of enzymes of the direct oxidative pathway in mammalian tissues and tumours. *Biochem. J.,* **56**: 171–175.

Hoar, William S., 1955. Seasonal variations in the resistance of goldfish to temperature. *Trans. Royal Soc. Canad.,* **49**: 25–34.

Hoar, William S., 1956. Photoperiodism and thermal resistance of goldfish. *Nature,* **178**: 364–365.

Kaplan, J. G., 1954. The alteration of intracellular enzymes. II: The relation between the surface and the biological activities of altering agents. *J. Gen. Physiol.,* **38**: 197–211.

Kaplan, J. G., 1955. The alteration of intracellular enzymes. I. Yeast catalase and the Euler effect. *Exp. Cell Res.,* **8**: 305–328.

Kaplan, J. G., and Wood-Ki Paik, 1956. The alteration of intracellular enzymes. IV. Kinetics of catalase alteration induced by chemical agents. *Canad. J. Biochem. Physiol.,* **34**: 25–38.

Kelly, T. L., E. D. Nielson, R. B. Johnson and C. S. Vestling, 1955. Glucose-6-phosphate dehydrogenase of adrenal tissue. *J. Biol. Chem.,* **212**: 545–554.

Krebs, H. A., 1950. Body size and tissue respiration. *Biochem. Biophys. Acta,* **4**: 249–269.

Patau, K., 1943. Zur statischen Buerteilung von Messungereihen (Eine neue t-Tafel). *Biol. Zentralbl.,* **63**: 152–168.

Peiss, C. N., and J. Field, 1950. The respiratory metabolism of excised tissues of warm- and cold-adapted fishes. *Biol. Bull.,* **99**: 213–224.

Precht, H., 1951. Der Einfluss der Temperatur auf die Atmung und auf einige Fermente beim Aal (*Anguilla vulgaris* L.). *Biol. Zentralbl.,* **70**: 71–85.

Precht, H., 1953. Über ruhestadien erwachsener Insekten. I. Versuche an Kartoffelkäfern (*Leptinotarsa decemlineata* Say). *Zeitschr. vergl. Physiol.,* **35**: 326–343.

Precht, H., J. Christophersen and H. Hensel, 1955. Temperatur und Leben. Springer, Berlin.

Robbie, W. A., 1948. Use of cyanide in tissue respiration studies. *Meth. Med. Res.,* **1**: 307–316.

Siegel, S., 1956. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill Book Co., New York.

Suhrman, R., 1955. Weitere Versuche über die Temperatur Adaptation der Karauschen (*Carrassius vulgaris* Nils). *Biol. Zentralbl.,* **74**: 432–448.

STABILITY OF THE CHROMATOPHOROTROPINS OF THE DWARF CRAYFISH, CAMBARELLUS SHUFELDTI, AND THEIR EFFECTS ON ANOTHER CRAYFISH[1]

MILTON FINGERMAN AND MILDRED E. LOWE

Department of Zoology, Newcomb College, Tulane University, New Orleans 18, Louisiana

The chromatophore systems of three crayfishes have been investigated to a considerable degree. Of these most is known about color changes in the dwarf crayfish, *Cambarellus shufeldti.*

Brown and Meglitsch (1940) found that the sinus gland in the eyestalk of the crayfish *Orconectes immunis* contained a chromatophorotropin that concentrated red pigment. McVay (1942) demonstrated that the central nervous system of this crayfish produced a material that concentrated red pigment.

The chromatophore system of the dwarf crayfish has been the subject of previous investigations by Fingerman (1957a, 1957b) and Fingerman and Lowe (1957a, 1957b). The red pigment dispersed maximally when dwarf crayfish were put on a black background and concentrated maximally when the crayfish were put on a white background. In the supraesophageal ganglia, circumesophageal connectives, and tissues of the eyestalks were chromatophorotropins that dispersed as well as concentrated red pigment (Fingerman, 1957a).

Behavior of red pigment on isolated portions of the carapace of *Cambarellus* that had been on black and on white backgrounds was also investigated (Fingerman, 1957b). Red pigment had an inherent tendency to concentrate nearly maximally when the chromatophores were no longer controlled by hormones. Reciprocal blood transfusions between specimens of *Cambarellus* that had been on black and on white backgrounds for two hours revealed that the blood always contained pigment-dispersing and -concentrating hormones. The degree of pigment dispersion at any time appeared to be due to the relative quantity of each antagonist in the blood.

Fingerman and Lowe (1957a) determined the effects of maintenance of specimens of *Cambarellus* on black and on white backgrounds for periods up to three weeks. The rates of red pigment dispersion and concentration in intact crayfish decreased progressively. In addition, red pigment gradually lost its inherent ability to concentrate after isolation.

The titers of chromatophorotropins in the circumesophageal connectives of dwarf crayfish that had been on a black or a white background for two weeks changed significantly relative to the titers in crayfish that had been on the same shade of background for two hours (Fingerman and Lowe, 1957a). They found that the hormone not needed for proper adaptation to one of the backgrounds, *e.g.,* red pigment-dispersing hormone of crayfish on a white background, was stored in the circumesophageal connectives. The quantities of chromatophorotropins in the blood also

---

[1] This investigation was supported by Grant No. B-838 from the National Institutes of Health.

changed. The hormone that was stored in the circumesophageal connectives of crayfish kept on a black or a white background for two weeks decreased in the blood and the hormone needed in the blood for background adaptation increased. These data provided evidence that the secretory products of the central nervous system were physiologically involved in the color change process.

Fingerman and Lowe (1957b) suggested that the chromatophorotropins in the eyestalks of dwarf crayfish were different from those in the circumesophageal connectives. Evidence was based on differences in rates of disappearance of the hormones in extracts of the eyestalks and circumesophageal connectives maintained at room temperature.

Chromatophorotropins that dispersed and concentrated red pigment were found in the eyestalks, supraesophageal ganglia, and circumesophageal connectives of the crayfish *Orconectes clypeatus* by Fingerman (1958). When specimens of *Orconectes clypeatus* were kept on a black background their red pigment dispersed maximally but when the crayfish were transferred to a white pan this pigment concentrated to an intermediate state only. Maximal red pigment concentration did not occur in specimens kept under constant illumination on a white background for 32 days. Hormones that dispersed red pigment could be demonstrated only by direct application of extracts to chromatophores on isolated portions of carapace. Direct injection of extracts did not produce red pigment dispersion.

The current concept of the origin of chromatophorotropins is that they are all neurosecretory products. Those found in the sinus gland originate in the medulla terminalis X organ as granules which migrate through axons to the sinus gland where they are stored (Bliss, Durand and Welsh, 1954). Fingerman and Aoto (unpublished observations) have found cytological evidence of neurosecretion in the supraesophageal ganglia, circumesophageal connectives, and optic ganglia of dwarf crayfish.

The present investigation was undertaken to shed light on several questions that have arisen as a result of the studies summarized above. For example, (1) since direct injection of tissue extracts of *Orconectes* into specimens of *Orconectes* did not cause pigment dispersion, we wished to learn whether tissue extracts from *Cambarellus* could disperse red pigment in specimens of *Orconectes* and vice versa. (2) The possibility that the chromatophorotropins found in the eyestalk are different from those found in the supraesophageal ganglia and circumesophageal connectives was also investigated further.

Materials and Methods

Adult specimens of the dwarf crayfish, *Cambarellus shufeldti,* and immature specimens of the crayfish *Orconectes clypeatus* were collected at Hickory, Louisiana, for use in the experiments described below. The specimens of both species used in the experiments were about 20 mm. long. The crayfishes were kept in aquaria that contained tap water approximately one inch deep. Air-conditioning kept the temperature of the laboratory between 25 and 27° C. The dark red chromatophores in the portion of the carapace dorsal to the heart were staged according to the system of Hogben and Slome (1931) by the senior author. Stage 1 represented maximal concentration of pigment, stage 5 maximal dispersion, and stages 2, 3, and 4 the intermediate conditions.

Tissue extracts were routinely prepared as follows. The organs to be assayed were dissected out and placed in Van Harreveld's solution (Van Harreveld, 1936). When the desired number of each organ was available the organs were transferred with a minimum of saline to a glass mortar, triturated, and suspended in a sufficient volume of Van Harreveld's solution to yield the desired concentration.

Crayfish that received injections of extracts had one eyestalk removed at least 12 hours prior to the experiment. Brown, Webb and Sandeen (1952) and Fingerman (1957a) found that the responses of one-eyed individuals to chromatophorotropins were greater than the responses of intact specimens, presumably because the presence of both eyestalks made the organisms more capable of antagonizing injected hormones.

Brown and Meglitsch (1940) and McVay (1942) were forced to use large specimens of *Orconectes immunis* whose exoskeleton was opaque. These investigators, therefore, applied extracts directly to the chromatophores on portions of the carapace removed from the body. The specimens used herein were small enough so that their carapaces were sufficiently transparent to allow accurate, direct observation of the underlying chromatophores.

For convenience and to save space the hormone that concentrated red pigment will be referred to as RPCH (red pigment-concentrating hormone) and that which dispersed red pigment as RPDH (red pigment-dispersing hormone). Use of the same letters for hormones in the eyestalks and in the supraesophageal ganglia plus the circumesophageal connectives does not imply that the chromatophorotropins have the same molecular structure.

Experiments and Results

Analysis of variation to be expected when the same extract is injected into two groups of dwarf crayfish

The aim of this experiment was to determine how much of the variance between two sets of data in the results presented below would be due to differential response of the crayfish used for assay and how much to differences in titers of chromatophorotropins in the extracts. To solve this problem an extract of the supraesophageal ganglia, with the circumesophageal connectives attached, of *Cambarellus* was prepared with a final concentration of one-third of a complement per 0.02 ml. Van Harreveld's solution. Ten one-eyed dwarf crayfish were then placed into each of two white enameled pans containing tap water; five were then placed into a third white pan. In like manner 25 were distributed among three black enameled pans. The crayfish in the white pans had been on a white background for at least one hour and were inspected prior to the experiment to be certain that their red pigment was maximally concentrated (stage 1). Crayfish in black pans had maximally dispersed red pigment (stage 5).

The crayfish in the two pans with five crayfish were each injected with 0.02 ml. Van Harreveld's solution as a control. The crayfish in the remaining pans were then injected with the extracts. Each crayfish in one white and one black pan was injected from the same syringe with 0.02 ml. of extract. The average stage of the red chromatophores of the crayfish in each pan was determined 15, 30, 60, 90 and 120 minutes from the time of injection.

The results are presented in Figure 1. As evident from inspection of the figure, RPCH and RPDH were present. The maximal effect of the former preceded that of the latter, the typical situation with extracts of supraesophageal ganglia and circumesophageal connectives (Fingerman, 1957a). The results of the two groups that received extracts were extremely similar, if not identical, since the difference between two consecutive determinations of the average chromatophore stage of the same group of crayfish may be 0.2 of a unit, but never more. The sum of the differences between the total of the average chromatophore indices for corresponding groups of crayfish was 0.6 unit for dispersing activity and 0.1 unit for concentrating activity. Results appreciably different from these values may, therefore, be considered as due to a significant difference in the quantities of chromatophorotropins in the extracts themselves and not to the crayfish used for the assay.

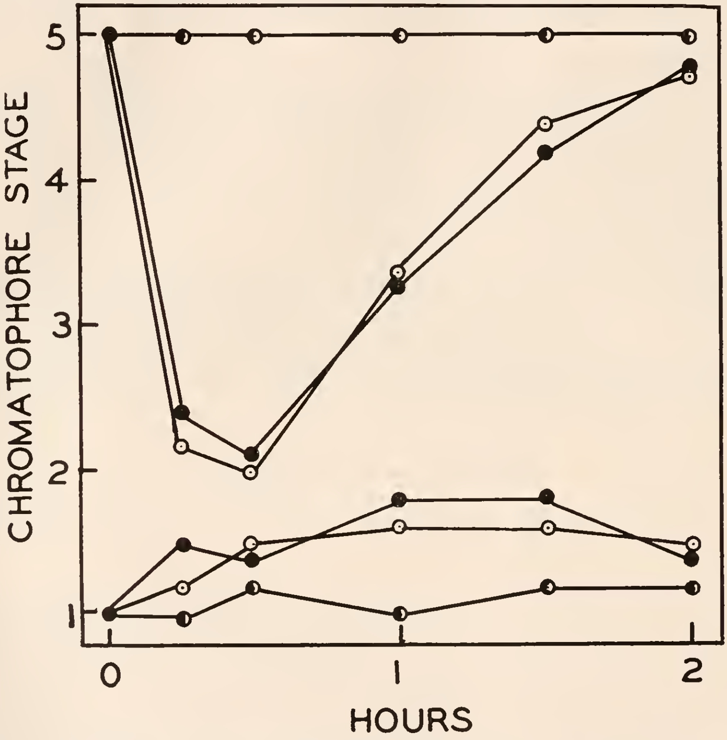


FIGURE 1. Responses of red chromatophores of one-eyed *Cambarellus* on black and on white backgrounds to the same extract of supraesophageal ganglia, with the circumesophageal connectives attached. Dots and circles, two groups that received the same extract; half-filled circles, control.

Responses of dwarf crayfish to chromatophorotropins of Cambarellus and Orconectes

The aim of this experiment was to compare the effects of extracts of eyestalks and supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish and *Orconectes* upon the dark red chromatophores of dwarf crayfish.

Since direct injection of freshly prepared extracts of eyestalks and supraesophageal ganglia plus the circumesophageal connectives of *Orconectes* into specimens of *Orconectes* never produced red pigment dispersion, we wished to learn whether these extracts could disperse red pigment in specimens of *Cambarellus*.

Both eyestalks and the supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish and *Orconectes* were removed and extracted as described above, so that the final concentration was one-third of a complement per 0.02 ml. Five one-eyed dwarf crayfish with maximally concentrated red pigment were placed into each of five white pans that contained aerated tap water. In like manner five one-eyed crayfish with maximally dispersed red pigment were placed into each of five black pans. The crayfish were injected and the chromatophores staged in the manner described in the first experiment.

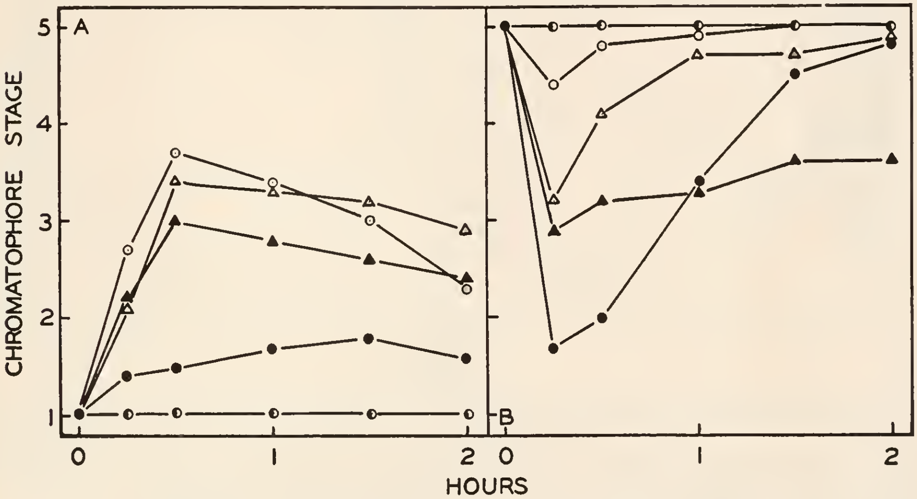


FIGURE 2. Responses of the red chromatophores of one-eyed *Cambarellus* on a white background (A) and on a black background (B) to extracts of eyestalks and supraesophageal ganglia, with the circumesophageal connectives attached, of *Cambarellus shufeldti* and *Orconectes clypeatus*. Half-filled circles are the controls. Open symbols are eyestalks, closed symbols nervous tissue. Circles, tissue of *Cambarellus;* triangles, tissue of *Orconectes*.

The results are presented in Figure 2 where each point represents the average of 10 crayfish. As evident from the figure, each extract contained RPCH and RPDH. The eyestalks of dwarf crayfish and *Orconectes* contained more RPDH and less RPCH than the supraesophageal ganglia plus the circumesophageal connectives. Significantly, the eyestalks and supraesophageal ganglia plus the circumesophageal connectives of *Orconectes* contained sufficient RPDH to produce an appreciable response in dwarf crayfish but no red pigment dispersion occurred when extracts of these tissues were injected into specimens of *Orconectes* (Fingerman, 1958).

Responses of Orconectes to chromatophorotropins of dwarf crayfish and Orconectes

Since direct injection of extracts of eyestalks and supraesophageal ganglia plus the circumesophageal connectives of *Orconectes* into dwarf crayfish did produce red pigment dispersion, we wished to determine the results of the reciprocal experiment. Red pigment of the specimens of *Orconectes* used in the assay was in an intermediate state of dispersion since red pigment in this species does not concentrate maximally when specimens are placed on a white background (Fingerman, 1958).

The results are presented in Figure 3 where each point represents the average of 10 crayfish. Extracts of tissues from *Orconectes* did not produce red pigment dispersion; RPCH alone was evident. As had been found previously (Fingerman, 1958), supraesophageal ganglia and circumesophageal connectives were more potent sources of RPCH than eyestalks.

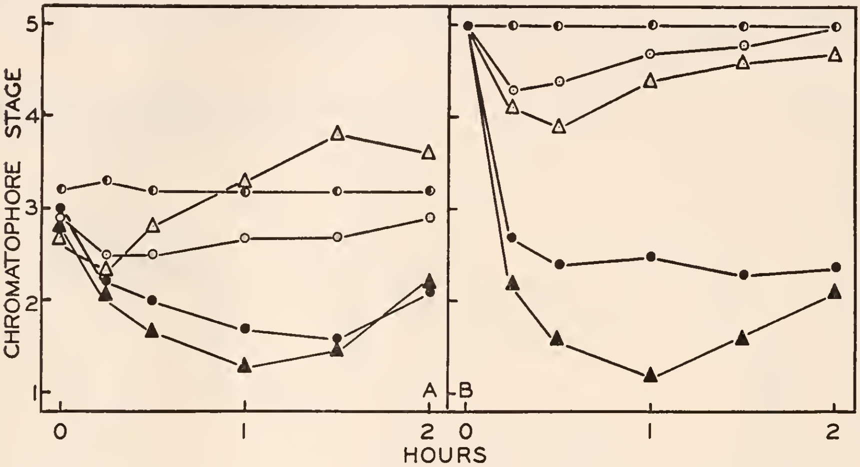


FIGURE 3. Responses of the red chromatophores of one-eyed *Orconectes* on a white background (A) and on a black background (B) to extracts of eyestalks and supraesophageal ganglia, with the circumesophageal connectives attached, of *Orconectes clypeatus* and *Cambarellus shufeldti*. Half-filled circles are the control. Open symbols are eyestalk, closed symbols nervous tissue. Circles, tissue of *Orconectes;* triangles, tissue of *Cambarellus.*

The responses of specimens of *Orconectes* on black and on white backgrounds to extracts of supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish and *Orconectes* were qualitatively similar. Concentration but no dispersion of red pigment in *Orconectes* was evident in spite of the fact that RPCH and RPDH were present in these extracts (Fig. 2).

Eyestalk extracts of *Cambarellus,* however, caused a transitory concentration of red pigment in *Orconectes* that was followed by dispersion. Concentration must have been caused by the small amount of RPCH in the eyestalks of dwarf crayfish and dispersion by the large amount of RPDH shown in Figure 2. Extracts of eyestalks of both species produced equal red pigment dispersion in dwarf crayfish but the eyestalk extract of *Orconectes* contained much more RPCH (Fig. 2). This difference was probably a contributing factor to the lack of red pigment dispersion in *Orconectes* with extracts of tissues from *Orconectes,* because the large amount of RPCH would hinder the expression of RPDH whereas in the eyestalk of *Cambarellus* the titer of RPDH so overbalanced the titer of RPCH that dispersion could not occur. The failure of extracts of *Orconectes* to disperse red pigment in *Orconectes* suggests that the titers of chromatophorotropins in the blood at the time of injection are important or else when the extracts are injected materials are secreted to antagonize the added hormones.

Influence of time and temperature upon chromatophorotropins of dwarf crayfish

This experiment was designed to determine the effects of boiling and maintenance at room temperature upon the titers of RPCH and RPDH in the supraesophageal ganglia with the circumesophageal connectives attached of dwarf crayfish. Twenty organs were removed and extracted in 1.2 ml. Van Harreveld's solution. The extract was then divided into two equal portions. One fraction was placed in boiling water for 30 seconds and cooled to room temperature. The boiled and unboiled extracts were then assayed on specimens in black and in white pans. The extracts were kept in the syringes on a table top at room temperature for 120 minutes and then assayed again.

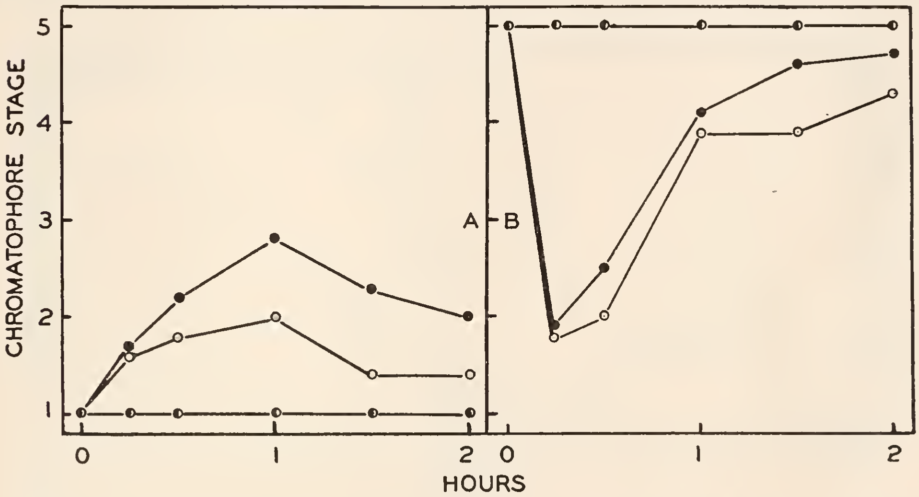


Figure 4. Responses of the red chromatophores of one-eyed *Cambarellus* on white (A) and on black (B) backgrounds to boiled (dots) and unboiled (circles) portions of the same extract of supraesophageal ganglia, with the circumesophageal connectives attached, of *Cambarellus* injected immediately after preparation. Half-filled circles, control.

The results are presented in Figures 4 (zero time injection) and 5 (120 minute injection). Boiling appeared to activate an inactive form of RPDH but had little, if any, effect on RPCH. Even more striking differences were apparent between the responses of the crayfish to boiled and unboiled extracts kept for 120 minutes at room temperature. The responses of the crayfish injected with the unboiled aged extract were the same as those observed by Fingerman and Lowe (1957b). The amount of RPCH had decreased considerably and the amount of RPDH had increased. In contrast, RPDH in the boiled preparation disappeared at a faster rate than RPCH. The latter showed no decreased potency. If anything, a slight increase was evident, probably due to the degeneration of its antagonist.

These results can be explained by assuming that RPDH exists in the neurosecretory cells in a functional form and a non-functional one which may be activated either by boiling or being kept at room temperature for two hours. Activation probably represents release of bound hormone from the interior of neurosecretory granules as has been shown with catechol amines by Hillarp and Nilson (1954) and Blaschko, Hagen and Welch (1955). Decrease in the quantity of the active form because of its instability occurs simultaneously with release of additional hormone from the neurosecretory granules in unboiled extracts. Inactivation of RPDH would occur in boiled extracts but no further release from the granules because boiling caused the immediate release of all bound RPDH from the neurosecretory granules. To illustrate, at the start of the experiment boiled extract caused more red pigment dispersion than unboiled (Fig. 4A). During the two hours at room temperature RPDH was becoming inactivated. Since in the boiled extract no inactive hormone would be available, degeneration alone occurred so that the net effect was decreased potency (Fig. 5A). In the case of the unboiled extract, during the two hours at room temperature the active form was being released from the neurosecretory granules at a faster rate than the functional hormone was becoming inactivated so that the net effect was an increased titer of RPDH. The same logic apparently does not apply to RPCH. Boiling seemed to stabilize the molecule so that it was not inactivated as was RPCH of unboiled extracts.

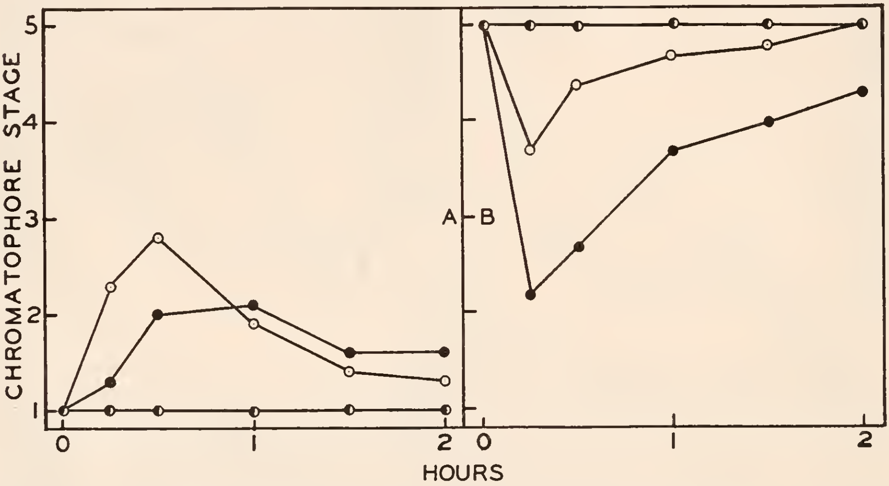


FIGURE 5. Responses of the red chromatophores of dwarf crayfish on white (A) and on black (B) backgrounds to the same extracts of Figure 4 but injected after 120 minutes at room temperature. See Figure 4 for key to symbols.

Stability of chromatophorotropins in dried tissue

The object of this experiment was to determine the effects of drying for two hours on RPCH and RPDH to rule out the possibility that the results of the previous experiment were due to the use of triturated tissues and not to the nature of the neurosecretory granules or the hormones. Supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish were dissected out and cut in half longitudinally. One batch was triturated immediately and re-suspended in sufficient Van Harreveld's solution to yield a final concentration of one-third of a complement per 0.02 ml. The second set was dispersed in the bottom of a glass mortar and allowed to dry at room temperature for 120 minutes, then triturated and suspended in sufficient Van Harreveld's solution for a final concentration of one-third of a complement per 0.02 ml. The freshly prepared extract was assayed and two hours later the extract of the dried tissues was assayed.

The results are presented in Figure 6 where each point represents the average of 10 crayfish. The results of drying the tissue for two hours were qualitatively the same as those observed with extracts left at room temperature for two hours. The extracts of dried tissues contained more RPDH and less RPCH than the extracts of fresh tissue. The same result was obtained when the extracts were left at room temperature for two hours. Therefore, the changes depicted in Figures 4 and 5 were not due solely to the aqueous environment of the extract but were mainly due to the instability of the hormones and probably of the neurosecretory granules in which the hormones are present when first formed.

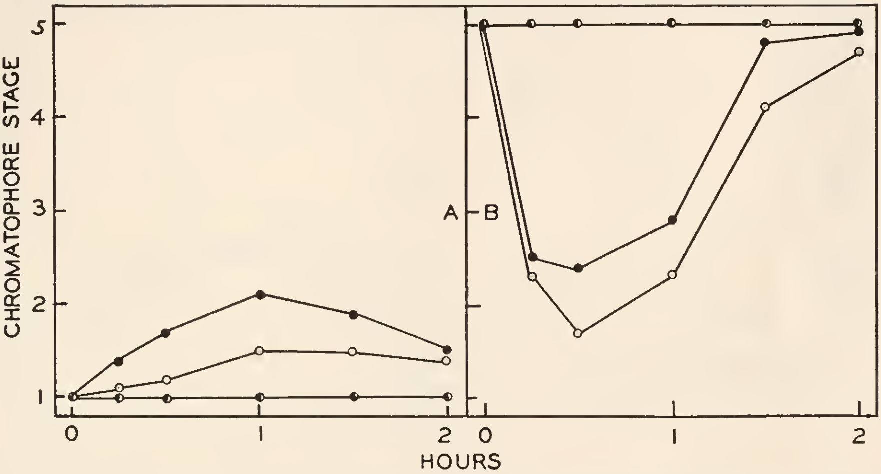


FIGURE 6. Responses of the red chromatophores of one-eyed dwarf crayfish on white (A) and on black (B) backgrounds to extracts of supraesophageal ganglia, with the circumesophageal connectives attached of *Cambarellus*. Circles, extracted at time of removal; dots, dried at room temperature for 120 minutes; half-filled circles, control.

Fractionation of chromatophorotropins in the sinus glands and supraesophageal ganglia plus the circumesophageal connectives of dwarf crayfish

The object of this experiment was to determine the relative solubilities of RPDH and RPCH from the sinus gland and the supraesophageal ganglia plus the circumesophageal connectives in absolute ethyl alcohol. In this way further information concerning the similarity of the corresponding molecules from the two sources would be obtained since Fingerman and Lowe (1957b) had obtained preliminary evidence that RPDH and RPCH in the eyestalks were different from the materials in the circumesophageal connectives of dwarf crayfish with the same functions.

The method employed was described in detail by Fingerman (1956). Six supraesophageal ganglia, with the circumesophageal connectives attached, were placed on a glass slide. The excess moisture was removed and the tissue was then smeared with a glass pestle. In like manner, six sinus glands were removed with a minimum of other tissues from eyestalks and smeared on a glass slide. The hormones in the smeared tissues were then extracted by dropping one ml. 100% ethyl alcohol from a syringe onto the tilted slide and collecting the alcohol in a watch glass. The alcohol was allowed to evaporate and the residue was re-suspended in 0.24 ml. Van Harreveld's solution.

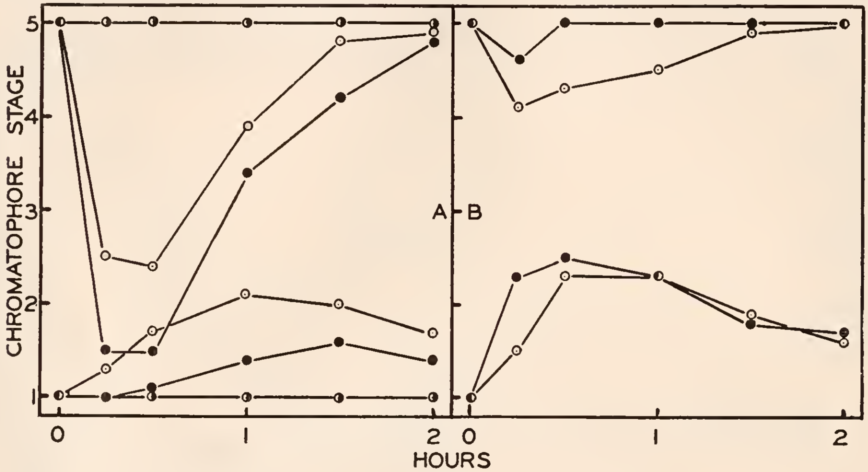


FIGURE 7. Responses of the red chromatophores of dwarf crayfish on black and on white backgrounds to extracts of supraesophageal ganglia, with the circumesophageal connectives attached (A), and sinus glands (B). Dots, alcohol-soluble fraction; circles, alcohol-insoluble fraction; half-filled circles, control.

After the few remaining drops of alcohol had evaporated from the glass slide the tissues were again extracted by placing 0.24 ml. Van Harreveld's solution on the tissues for two to three minutes. The fluid was then taken up in a syringe. The four fractions were then assayed.

The results are presented in Figure 7 where each point represents the average of 10 crayfish. As evident from the left half of the figure (nervous tissue), the alcohol-soluble fraction contained more RPCH and less RPDH than the alcohol-insoluble fraction. Consideration of the results obtained with the sinus glands revealed the reverse situation prevailed. The conclusion may, therefore, be drawn that the chromatophorotropins of the supraesophageal ganglia plus the circumesophageal connectives are different from those of the sinus gland.

Enzymatic inactivation of chromatophorotropins of Cambarellus

This set of experiments was designed to shed further light on what happens to chromatophorotropins after they have been secreted into the blood. Carstam

(1951) showed that the hypodermis of the prawn *Leander adspersus* contains an enzyme capable of inactivating chromatophorotropins from the sinus gland. Fingerman and Lowe (1957b) showed that the presence of hypodermis of dwarf crayfish likewise hastened the inactivation of chromatophorotropins in nervous tissue. We decided, therefore, to investigate further the nature of this inactivation.

For the first experiment of the series supraesophageal ganglia, with the circumesophageal connectives attached, were removed from dwarf crayfish and triturated with a sufficient volume of Van Harreveld's solution to yield 0.90 ml. extract with a concentration of one-third complement per 0.02 ml. Two-hundredths ml. of this extract was then injected into each of five one-eyed crayfish on a white background and into a like number on a black background. The entire carapace had, in the meantime, been removed from six dwarf crayfish. Three of the carapaces were boiled to destroy the proteins in the hypodermis. The six carapaces were then placed on individual glass depression slides. Into each depression containing a carapace was placed 0.1 ml. of the extract. The slides were then covered to minimize evaporation. After the extract had been exposed to the carapaces for two hours, the fractions that had been exposed to boiled and unboiled carapace were collected separately and assayed. The results are presented in Figure 8 where each point represents the average of 10 crayfish.

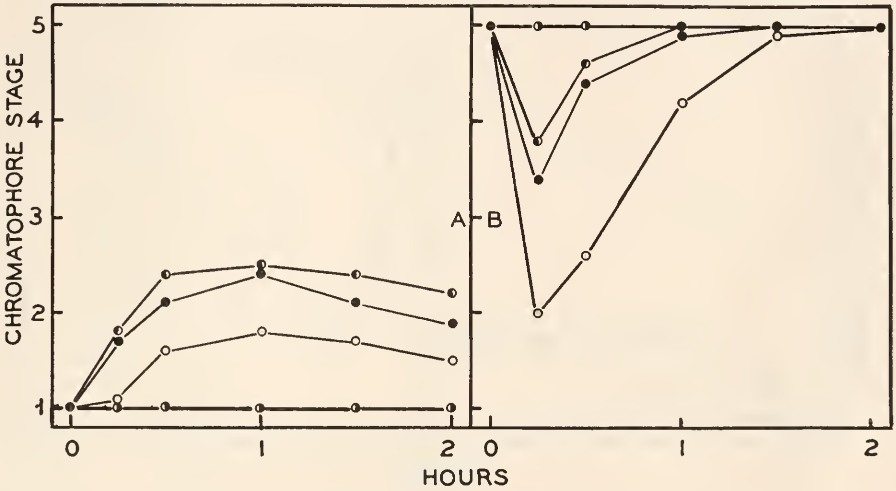


FIGURE 8. Responses of the red chromatophores of *Cambarellus* on white (A) and on black (B) backgrounds to unboiled extracts of supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish. Circles, extract administered immediately after boiling; dots, after two hours exposure to boiled carapace; circles filled on left side, after two hours exposure to unboiled carapace; circles filled on right side, control.

The unboiled extract exposed to the boiled and unboiled pieces of carapace behaved just as the unboiled extract shown in Figures 4 and 5. After two hours of exposure to both boiled and unboiled carapace the unboiled extract showed an increase in red pigment-dispersing potency and a decrease in RPCH. The same results were obtained when the unboiled extract was simply left at room temperature for two hours. The extract that had been exposed to unboiled carapace, however, contained more RPDH and less RPCH than the extract that had been in contact with boiled carapace. Presumably, this difference was due to preferential inactivation of RPCH by an enzyme in the hypodermis of the unboiled carapace. This enzyme must have been destroyed in the boiled carapaces. Apparently, the enzyme destroyed RPCH in preference to RPDH and for this reason the latter was more abundant after two hours of exposure. Spontaneous release of active RPDH from the neurosecretory granules also must have occurred during the two hours of exposure to the carapace.

Since boiled extract kept for two hours had less RPDH than was present at the time of heating (Figs. 4 and 5), the logical sequel to the experiment just described wherein inactivation of the hormones was not great, was to expose boiled extracts to boiled and unboiled carapaces for two hours. Furthermore, as mentioned above, the titer of RPCH in boiled extracts showed no tendency to decrease for at least two hours at room temperature so that any change in the titer of this hormone would have been enzymatically induced.

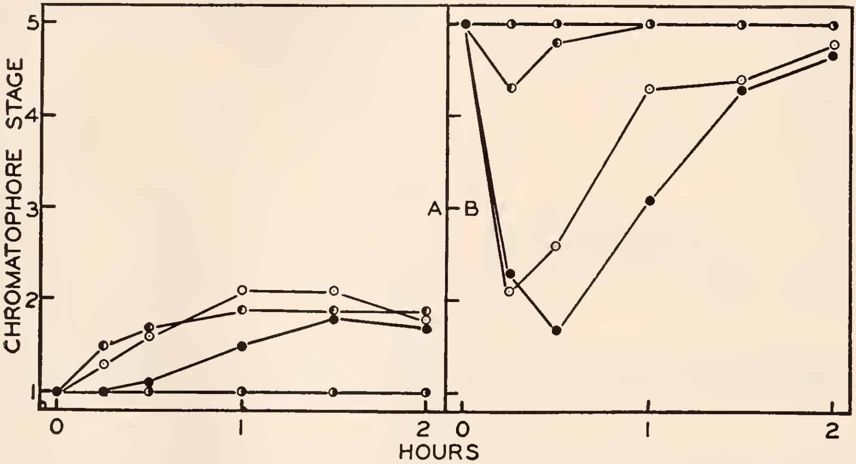


Figure 9. Responses of the red chromatophores of *Cambarellus* on white (A) and on black (B) backgrounds to boiled extracts of supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish. Circles, extract administered immediately after boiling; dots, after two hours exposure to boiled carapace; circles filled on left side, after two hours exposure to unboiled carapace; circles filled on right side, control.

Extracts for this experiment were prepared and handled in the same manner as those for the first experiment of this series with the one exception that the extract was immersed in boiling water for 30 seconds, cooled, and then placed on the depression slides. The results of the second experiment are shown in Figure 9. Each point represents the average of 10 crayfish.

The extract exposed to boiled carapace for two hours behaved exactly like the boiled extract of Figure 5; the titer of RPDH decreased markedly whereas no tendency for RPCH to diminish was apparent. However, the results with the boiled extract exposed to unboiled carapace were quite different from those obtained with extracts exposed to unboiled carapace. The quantity of RPCH decreased markedly, presumably due to enzymatic inactivation. The quantity of RPDH showed little or no decrease, probably because so much of its antagonist was destroyed that a lower titer of RPDH was able to exert a sizeable effect. Here also a preferential inactivation of RPCH occurred.

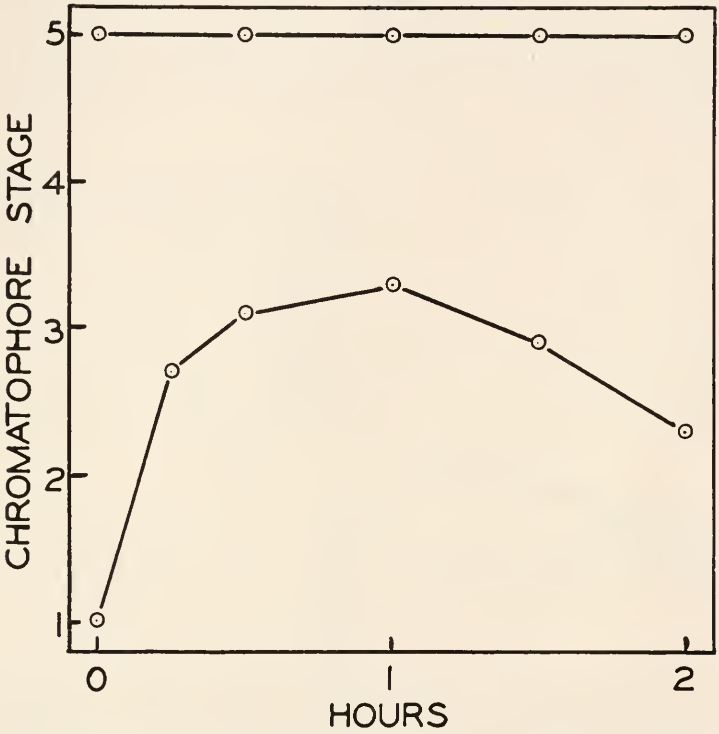


Figure 10. Responses of the red chromatophores of dwarf crayfish on black and on white backgrounds to epinephrine.

Responses of the red chromatophores of dwarf crayfish to epinephrine

An epinephrine solution (adrenalin chloride) manufactured by Parke, Davis and Co. was diluted with Van Harreveld's solution so that the final concentration was 2×10^{-5} gram epinephrine per 0.02 ml. Ten one-eyed dwarf crayfish with maximally concentrated red pigment and a like number with maximally dispersed red pigment were each injected with 0.02 ml. of the solution of epinephrine. The average stage of the red chromatophores of the crayfish in each pan was then determined 15, 30, 60, 90, and 120 minutes after they had been injected. The experiment was repeated once.

The results are presented in Figure 10 where each point represents the average of 20 crayfish. As is evident from inspection of the figure, epinephrine produced considerable pigment dispersion. No tendency for the red pigment to concentrate was apparent.

Filterability and adsorbability of the chromatophorotropic factors present in the supraesophageal ganglia and circumesophageal connectives of dwarf crayfish

An extract of the supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish was prepared so that the final concentration was one-third of a complement per 0.02 ml. of fluid. The extract was then divided into three portions. One part was untreated. The second aliquot was filtered once through "Aloe Standard American" filter paper, catalogue no. 42700. The third was shaken through crushed charcoal and then centrifuged to remove the small particles of charcoal. The fractions were then assayed. The experiment was done twice.

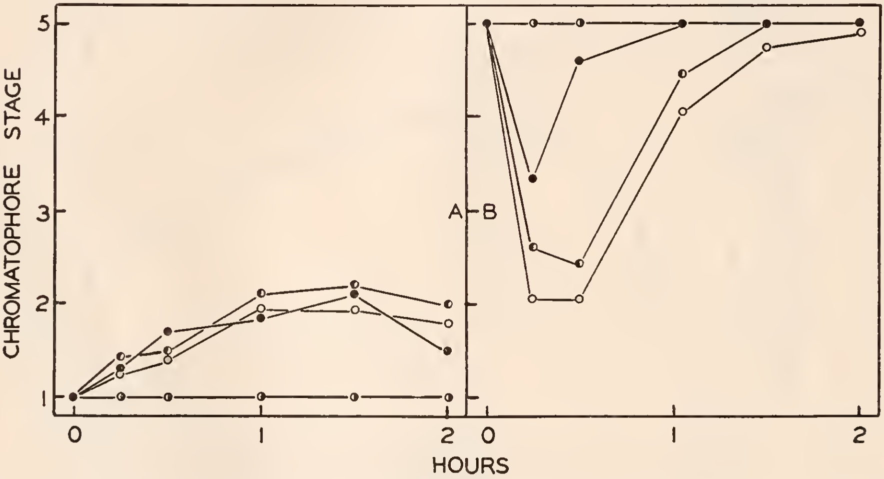


FIGURE 11. Responses of the red chromatophores of dwarf crayfish on white (A) and on black (B) backgrounds to extracts of supraesophageal ganglia, with the circumesophageal connectives attached. Circles filled on the left side, pure extract; circles, extract filtered through paper; dots, extract shaken with powdered charcoal; circles filled on right side, control.

The results are presented in Figure 11 where each point represents the average of 10 individuals. No striking differences were found among the titers of RPDH in the extracts. A slight adsorption of RPDH on filter paper may have occurred. In contrast, a striking decrease of RPCH in the extract shaken with charcoal was apparent whereas this substance was not adsorbed on filter paper. A slight augmentation of the titer of RPCH in the filtered extract was apparent although the reason for this is not clear since an appreciable quantity of its antagonist (RPDH) was still in the extract.

DISCUSSION

The experiments described above were based upon some problems that investigators of color changes in crustaceans have been considering for several years. One such is why an extract that concentrates pigment in the animal from which it was obtained disperses pigment when injected into another species. This problem is strikingly exemplified by the results obtained when extracts of *Orconectes* and dwarf crayfish were injected into the donor species as well as interspecifically. Extracts of tissues of specimens of *Orconectes* never produced red pigment dispersion in specimens of *Orconectes* yet they produced striking concentration and dispersion of the pigment of dwarf crayfish. On the other hand, extracts of the eyestalk of dwarf crayfish were capable of producing dispersion of red pigment in specimens of both *Orconectes* and dwarf crayfish.

Specimens of *Orconectes* may have an excellent feed-back mechanism associated with the chromatophore system so that any displacement of the red pigment in crayfish on a white background to a more dispersed state is rapidly met with release of RPCH to maintain a steady-state of the red pigment. Extracts of the eyestalks of dwarf crayfish alone were able to overcome this mechanism because of the large amount of RPDH present in the extract relative to RPCH. The other extracts used had more RPCH present relative to the quantity of RPDH so that the *Orconectes* could easily antagonize the RPDH in these extracts by secretion of some RPCH. In support of this hypothesis are the results of Fingerman (1958) who showed that the blood of *Orconectes* always contained RPCH and RPDH and that the state of the pigment at any given time appeared to be due to the relative quantity of each hormone in the blood. He was able to produce slight dispersion of red pigment in specimens of *Orconectes* with blood from specimens on a white background and considerable dispersion with blood from *Orconectes* on a black background.

The conclusive evidence presented herein that the RPCH and RPDH found in the sinus gland are different from the materials found in the supraesophageal ganglia plus the circumesophageal connectives that concentrate and disperse red pigment adds another complication to the already complicated study of control of color changes in crustaceans. The problem immediately arises why an organism should produce two hormones to accomplish the same function. Since the maximal red pigment-dispersing effect obtained when extracts of eyestalks or sinus glands are used occurs sooner than that of the supraesophageal ganglia plus the circumesophageal connectives (Figs. 2 and 7), RPDH of the sinus gland may be used to move the pigment rapidly to the desired state of dispersion and once at this state the factor of the supraesophageal ganglia plus the circumesophageal connectives may take charge. Such a situation may explain the results of Fingerman and Lowe (1957b) who assayed the amount of RPDH in the blood of dwarf crayfish after a background change and found that the amount in the blood 30 minutes after transfer of crayfish from a white to a black background was more than needed to maintain the pigment in the maximally dispersed condition. Perhaps the excess titer was due to RPDH from the sinus gland and once the pigment had become maximally dispersed the pigment was kept in this state by a lower titer of RPDH from the supraesophageal ganglia and the circumesophageal connectives.

Fingerman (1956) demonstrated that in the blue crab, *Callinectes sapidus,* the factor in the sinus gland that dispersed red pigment was different from the hormone in the circumesophageal connectives that accomplished the same effect. Perhaps this difference in hormones in the sinus gland and central nervous organs is a general thing among crustaceans.

Carstam (1951) showed that an enzyme that inactivated the red pigment concentrating hormone of the sinus gland of the prawn *Leander adspersus* was present in the hypodermis of the following crustaceans, *Leander adspersus, Cancer pagurus, Homarus vulgaris,* and *Idothea neglecta.* Presumably inactivation of RPCH of *Cambarellus* (Fig. 9) was due to the presence of this enzyme in the hypodermis of the unboiled carapace.

Results obtained by different investigators with mammalian hormones injected into crustaceans do not form a consistent pattern. Östlund and Fänge (1956) found that epinephrine dispersed red pigment in *Leander adspersus.* The same results were obtained with dwarf crayfish (Fig. 10). In contrast, Nagano (1950) found that adrenalin produced pigment concentration in the shrimp *Paratya compressa* and Abramowitz and Abramowitz (1938) found no effect of adrenalin on the chromatophores of the fiddler crab *Uca pugilator.* McVay (1942) found adrenalin ineffective on the red chromatophores of the crayfish *Orconectes immunis.*

The results presented above showed that RPCH of the supraesophageal ganglia plus the circumesophageal connectives is not removed by filtering through paper but is adsorbed on charcoal. McVay (1942) found the same results with extracts of the central nervous system of *Orconectes immunis.* She postulated that RPCH was present to some extent in combination with a non-active carrier. Furthermore, she postulated that neither the complex nor the dissociated form of the hormone was adsorbed on filter paper but that the complex would be adsorbed by charcoal but not the dissociated form. This postulated complex might consist of hormone molecules within neurosecretory granules. Evidence in support of this concept has been obtained for the black pigment-dispersing hormone of the fiddler crab *Uca pugilator* by Pérez-González (1957).

Summary and Conclusions

1. Hormones that concentrated and dispersed red pigment were present in the eyestalks and supraesophageal ganglia plus the circumesophageal connectives of the crayfishes *Orconectes clypeatus* and *Cambarellus shufeldti.*

2. Direct injection of extracts of eyestalks and supraesophageal ganglia plus the circumesophageal connectives of *Orconectes* into *Orconectes* produced red pigment concentration alone, whereas the same extracts dispersed as well as concentrated red pigment in *Cambarellus.* Extracts of eyestalks of *Cambarellus,* however, dispersed red pigment in *Orconectes.*

3. Boiled extracts of supraesophageal ganglia plus the circumesophageal connectives produced more dispersion of red pigment than unboiled extracts.

4. Maintenance of boiled and unboiled extracts of supraesophageal ganglia plus circumesophageal connectives of *Cambarellus* for two hours at room temperature yielded additional information about the stability of chromatophorotropins. Red pigment-dispersing hormone in unboiled extracts increased and concentrating effect decreased. In contrast, in boiled extracts no tendency for red pigment-concentrating hormone to disappear was evident whereas dispersing hormone decreased. The red pigment-dispersing hormone is probably present in heat-labile neurosecretory granules as well as in the free state.

5. The hormones in the sinus gland that dispersed and concentrated red pigment were different from the factors in the supraesophageal ganglia plus the circumesophageal connectives with the same functions.
6. Evidence was presented for an enzyme in the hypodermis that inactivates chromatophorotropins.
7. Epinephrine dispersed red pigment in specimens of *Cambarellus*.
8. The results were discussed in relation to the data of other investigators.

LITERATURE CITED

Abramowitz, A. A., and R. K. Abramowitz, 1938. On the specificity and related properties of the crustacean chromatophorotropic hormone. *Biol. Bull.,* **74**: 278–296.

Blaschko, H., P. Hagen and A. D. Welch, 1955. Observations on the intracellular granules of the adrenal medulla. *J. Physiol.,* **129**: 27–49.

Bliss, D. E., J. B. Durand and J. H. Welsh, 1954. Neurosecretory systems in decapod Crustacea. *Zeitschr. Zellforsch.,* **39**: 520–536.

Brown, F. A., Jr., and A. Meglitsch, 1940. Comparison of the chromatophorotropic activity of insect corpora cardiaca with that of crustacean sinus glands. *Biol. Bull.,* **79**: 409–418.

Brown, F. A., Jr., H. M. Webb and M. I. Sandeen, 1952. The action of two hormones regulating the red chromatophores of *Palaemonetes. J. Exp. Zool.,* **120**: 391–420.

Carstam, S. Ph., 1951. Enzymatic inactivation of the pigment hormone of the crustacean sinus gland. *Nature,* **167**: 321–322.

Fingerman, M., 1956. Physiology of the black and red chromatophores of *Callinectes sapidus. J. Exp. Zool.,* **133**: 87–106.

Fingerman, M., 1957a. Endocrine control of the red and white chromatophores of the dwarf crawfish, *Cambarellus shufeldti. Tulane Stud. Zool.,* **5**: 137–148.

Fingerman, M., 1957b. Physiology of the red and white chromatophores of the dwarf crayfish, *Cambarellus shufeldtii. Physiol. Zoöl.,* **30**: 142–154.

Fingerman, M., 1958. The chromatophore system of the crawfish *Orconectes clypeatus. Amer. Midl. Nat.,* in press.

Fingerman, M., and M. E. Lowe, 1957a. Influence of time on background upon the chromatophore systems of two crustaceans. *Physiol. Zoöl.,* **30**: 216–231.

Fingerman, M., and M. E. Lowe, 1957b. Hormones controlling the chromatophores of the dwarf crawfish, *Cambarellus shufeldti:* their secretion, stability, and separation by filter paper electrophoresis. *Tulane Stud. Zool.,* **5**: 149–171.

Hillarp, N. A., and B. Nilson, 1954. The structure of the adrenaline and noradrenaline containing granules in the adrenal medullary cells with reference to the storage and release of the sympathomimetic amines. *Acta Physiol. Scand.,* **29**: 251–263.

Hogben, L. T., and D. Slome, 1931. The pigmentary effector system. VI. The dual character of endocrine coordination in amphibian colour change. *Proc. Roy. Soc. Lond., Ser. B,* **108**: 10–53.

McVay, J. A., 1942. Physiological experiments upon neurosecretion with special reference to *Lumbricus* and *Cambarus.* Doctoral thesis, Northwestern University, Evanston, Illinois.

Nagano, T., 1950. Physiological studies on the pigmentary system of Crustacea. V. Drug action upon the pigmentary system of a shrimp. *Sci. Repts. Tohoku Univ., 4th Ser.,* **18**: 293–303.

Östlund, E., and R. Fänge, 1956. On the nature of the eye-stalk hormone which causes concentration of red pigment in shrimps (Natantia). *Annales des Sci. Nat. (Zool.),* **18**: 325–334.

Pérez-González, M. D., 1957. Evidence for hormone-containing granules in sinus glands of the fiddler crab *Uca pugilator. Biol. Bull.,* **113**: 426–441.

Van Harreveld, A., 1936. A physiological solution for fresh water crustaceans. *Proc. Soc. Exper. Biol. and Med.,* **34**: 428–432.

POTASSIUM AND SODIUM REGULATION IN AN INTERTIDAL CRAB

WARREN J. GROSS

Division of Life Sciences, University of California, Riverside, California

This paper will show that when the crab *Pachygrapsus crassipes* is exposed to a simple osmotic stress it regulates its blood sodium and potassium equally well. However, more potassium than sodium is exchanged between animal and external medium for a given alteration in the blood, which means that a source of potassium other than the blood is contributing to the exchanges. Also, evidence will be produced indicating that sources other than the blood contribute to sodium exchanges between crab and medium, thus suggesting the presence of adaptive salt pools, a phenomenon postulated by Hukuda (1932).

Much work has been done, especially on mammals, demonstrating that the ionic concentration in tissues can be altered by experimentally varying the concentration of ions in the environmental fluid. Such studies are reviewed by Manery (1954) and Harris (1956). Krogh (1939), Prosser *et al.* (1950) and Beadle (1957) consider the subject of ionic and osmotic regulation in aquatic animals, but these reviews are concerned chiefly with changes in the blood which are effected by alterations in the concentration of the external medium. Little information is available concerning the fate of ions entering an aquatic animal from a hypertonic external medium, nor the source of ions leaving an animal to a hypotonic medium. Prosser *et al.* (1955) have demonstrated the final sodium and potassium blood concentration of the crab *Pachygrapsus crassipes* after it is exposed to diluted or concentrated sea water. However, the total exchange of these two ions between animal and medium has not been shown. Thus, it could not be stated, for example, whether the loss of blood ions by an animal to a hypotonic medium represented the total loss by the animal or whether sources other than the blood were contributing to the loss.

MATERIALS AND METHODS

The shore crab, *Pachygrapsus crassipes* Randall, used in this investigation was collected at Newport and Laguna, California. *Pachygrapsus* is particularly suitable for this type of study because it can regulate osmotically in dilute as well as concentrated sea water. Also, it can live out of water for extended periods (Jones, 1941; Prosser *et al.*, 1955; and Gross, 1955). All specimens used were between molts and were mature, none weighing less than 20 grams.

Sodium and potassium concentrations were measured by means of a Beckman flame photometer using a standard which contained potassium and sodium approximating the respective proportions in the blood. Samples of blood of approximately 0.05 ml. were extracted serially from individual crabs, measured, in calibrated capillary tubes and diluted in 25 ml. of distilled water. Such samples were used directly in the flame photometer. Known quantities of sodium added to the blood thus treated could be recovered within 2% with concentrations of about 500 mEq./l. and potassium could be recovered within 10% of concentrations of around 10 mEq./l.

The exchange of ions between animal and external medium was determined as follows: Blood from a crab recently removed from sea water of known concentration was analyzed for sodium and potassium. The same specimen was kept out of water for a brief time to permit the rapid coagulation of blood and then was immersed in a known volume of a different salinity having been rinsed first in that new salinity. The volume of medium varied from 50 ml. to 100 ml., depending on the size of the specimen. All animals could raise themselves out of the water and thus usually were not immersed completely. After a period of immersion (24–48 hours) during which time adequate precautions were taken against evaporation, the crab was removed, and its blood as well as external medium were analyzed again for potassium and sodium. Thus determinations of sodium and potassium were made on crabs both before and after exposure to the experimental media. The following experimental treatments were studied:

a) Transferred from normal sea water to dilute sea water (25% or 50%).
b) Transferred from normal sea water to concentrated sea water (approximately 150%).
c) Acclimated for 1–2 days to 50% sea water, then transferred to approximately 150% sea water.
d) Acclimated for 1–2 days to approximately 150% sea water, then transferred to 50% sea water.

The effects of desiccation on the blood concentrations of sodium and potassium also were investigated. Blood from crabs freshly removed from normal sea water was analyzed for potassium and sodium; the crab was then blotted dry, weighed and placed in a chamber at 15° C. for desiccation. After a period ranging from 24 to 72 hours, the animal was dipped in sea water to replace the evaporated branchial fluid, blotted and weighed. The blood then was analyzed again for sodium and potassium. Blood from a few partially desiccated crabs was analyzed for potassium and sodium; then the animal was desiccated further, and its weight change between the two desiccated conditions was measured without dipping as above, since there was little or no water remaining in the branchial chamber after partial desiccation. Then the blood was analyzed again for sodium and potassium. Since the effects of desiccation on the ionic concentration of the blood per unit weight loss by evaporation were not significantly different for the two above methods, it can be concluded that branchial fluid is accurately replaced by dipping and the weight losses caused by evaporation do not include the branchial fluid. Thus the blood sodium or potassium concentration change could be determined for a given weight loss caused by evaporation.

All of the above treatments were endured by most of the crabs tested which seemed to recover when returned to normal sea water. The repeated blood sampling resulting in a total loss of not more than 0.2 ml. does not seem to impose too great a stress since a 30-gram crab containing about 5 to 10 grams of blood can survive the loss of 1 ml.

RESULTS

Table I, which presents the blood potassium and sodium concentrations of normal *Pachygrapsus,* freshly removed from the sea, shows the mean potassium concentration as 7.43 mEq./l. and the mean sodium concentration as 483.3 mEq./l. While the latter value is in close agreement with Prosser *et al.* (1955), the value for potassium is considerably less than 12.1 mEq./l. reported by the above workers.

TABLE I

Blood concentrations of crabs after treatment

Treatment		No. of crabs	Final blood concentration		Blood changes		
			Mean mEq./l.	S.D.	Mean mEq./l.	S.D.	% original
100% sea water to 25%* or 50% sea water	Na	28	373	37.0	−107	38.0	−22.8
	K	22	6.03	2.16	−2.25	2.11	−28.1
100% sea water to 150% sea water	Na	25	574	26.1	+99.2	26.1	+20.7
	K	24	9.71	1.54	+2.67	1.25	+38.3
50% sea water to 150% sea water	Na	12	572	37.2	+180	37.1	+43.5
	K	12	9.21	1.19	+3.26	0.90	+58.2
150% sea water to 50% sea water	Na	14	406	37.3	−174	61.2	−29.7
	K	14	6.78	1.35	−2.92	1.71	−29.2
		No. of crabs	Means		S.D.		
Normal crabs freshly removed from the sea	Na	36	mEq./l.	483.3	17.3		
			% sea water	103			
	K	36	mEq./l.	7.43	0.72		
			% sea water	74			

* Blood concentrations of crabs immersed in 25% sea water were not significantly different from those immersed in 50% sea water. This is because the former were immersed for briefer periods.

This difference is even more significant because the potassium values obtained in the present investigation are less than those in the external medium (9.8 mEq./l.) whereas the values of Prosser *et al.* were more than the medium. The latter studies were made at Pacific Grove, California, about 300 miles north of the Laguna area where specimens for the present investigation were collected. Possibly temperature is the significant difference.

Not only is the blood potassium of *Pachygrapsus* less concentrated than the potassium of normal sea water, the natural medium of this crab, but it remains less concentrated even when immersed in media as dilute as 50% sea water, *i.e.,* there is a tendency for the blood potassium to remain less concentrated than that in the medium. For 28 crabs immersed 24–48 hours in 50% sea water (salts were lost from the animal, increasing the medium concentration) the mean ratio, blood potassium (mEq./l.)/medium potassium (mEq./l.) was 0.897, S.D = 0.19 which is significantly less than one, $P < 0.01$. As expected, the blood potassium is likewise less concentrated in hypertonic media.

Blood sodium on the other hand, which contributes about half of the blood osmotic pressure, was maintained above the sodium concentration of dilute media and below sodium concentrations in concentrated media, indicating active regulation of this ion as described by Prosser *et al.* (1955). Thus under the conditions of these experiments neither blood sodium nor potassium achieves concentrations equal to those of the external medium. Neither does the blood become isotonic to the external medium (Jones, 1941; Prosser *et al.,* 1955; Gross, 1957).

Table I also shows the ionic alterations that occur under various treatments in aqueous media. These values are presented to demonstrate the magnitude of blood ion changes, but they should not be considered comparable to those values reported in other investigations where animals were immersed completely in large volumes of water. The prime objective of the present investigation is to demonstrate the ionic change that occurs in the medium per given ionic change in the blood of the animal. It should be pointed out that the dilution and concentration of the blood of *Pachygrapsus* such as is shown in Table I is effected by salt exchange, not water (Gross, 1957). This must mean that a loss of ions in the external medium is essentially the same as an injection of salts into the animal. On the other hand a gain of ions in the external medium is the same as a removal of ions from the animal. We cannot say at this point whether or not those exchanges occur only between blood and external medium.

Now the "apparent volume of distribution" (Winkler *et al.,* 1943) in the animal for each ion can be estimated from the following equation,

$$V = M/P \times 100,$$

where: V = "apparent volume of distribution" in % body weight;

$$M = \frac{\text{weight of medium}}{\text{weight of animal}};$$

$$P = \frac{\text{change in blood ion concentration (mEq./l.)}}{\text{change in medium ion concentration (mEq./l.)}}$$

(the observed ratios, P are presented in column 2, Table V, corrected to an M value of 1.0).

Table II shows the effect of desiccation on the blood of crabs. After a crab is desiccated, the "apparent volume of distribution" can be estimated by the equation,

$$V = \frac{E}{C_1/C_2\text{-}1},$$

TABLE II

Sodium and potassium increases in the blood caused by desiccation

	No. of crabs	Change in ion concentration (% original concentration) per 1% body weight loss*		
		Mean	S.D.	95% fiducial limits
Na	84	+2.20	0.71	2.05– 2.35
K	50	+8.68	11.75	5.36–12.00

* By evaporation.

where E = % weight change caused by evaporation,

C_1 = initial blood ion concentration (mEq./l.),

C_2 = final blood ion concentration (mEq./l.).

Table III gives the "apparent volume of distribution" for sodium for the various treatments. Here it can be seen that the volume in question varies with the treatment. Thus by the desiccation method it averages 48.9% body weight. When crabs were transferred from normal sea water to dilute sea water the volume av-

TABLE III

"Apparent volume of distribution" for sodium

	Treatment	No. of crabs	Volume in % body weight	S.D.
A	Desiccation	84	48.9	12.8
B	Normal sea water to Dilute sea water	28	39.0	12.8
C	Normal sea water to Concentrated sea water	25	37.9	11.7
B + C	Normal sea water to Dilute or concentrated sea water	53	38.5	12.1
D	150% sea water to 50% sea water	14	46.9	4.52
E	50% sea water to 150% sea water	12	44.8	8.56
D + E	50% or 150% sea water to 150% or 50% sea water	26	45.9	6.55

erages 39.0%; where animals were transferred from normal sea water to concentrated sea water, 37.9% body weight. The latter two means are not significantly different from each other, so the values for the two treatments were combined, and these averaged 38.5% body weight. This value was shown by "t" evaluation to be significantly different from the above 48.9% value estimated by the desiccation method, P = 0.003. When large blood changes were effected by first acclimating the animal to either dilute or concentrated sea water (50 or 150%), then transferring it to the opposite stress and measuring the resultant changes in the blood and external medium of the animal, the "apparent volume of distribution" averaged 45.9% body weight. There was no significant difference between values calculated on crabs started in 50% sea water and those initially placed in 150% sea water. However, the volume 45.9% body weight was shown to be significantly different from 38.5% body weight determined for crabs transferred from normal sea water to various stresses (B + C), P = 0.0004. Yet, the "apparent volume of distribution" determined by (A), the desiccation method (48.9% body weight), was not significantly different from 45.9% body weight.

TABLE IV

Relative sodium and potassium concentration changes in the blood caused by osmotic stress

	Treatment	$\frac{\text{Blood sodium change (\% original)}}{\text{Blood potassium change (\% original)}}$		
		No. of crabs	Mean of ratios	S.D.
A	100% sea water to 25% or 50% sea water	22	1.24	1.31
B	100% sea water to 150% sea water	23	0.72	0.57
A + B	100% sea water to dilute or concentrated sea water	45	0.97	0.91
C	50% sea water to 150% sea water	12	0.83	0.31
D	150% sea water to 50% sea water	14	0.92	0.22
C + D	50% or 150% sea water to 150% or 50% sea water	26	0.88	0.26

Table III reveals also that the variance in values for "apparent volume of distribution" is markedly reduced when the crab is exposed to extreme osmotic stresses (*e.g.*, transferring from 50% to 150% sea water). This merely means that a large sodium change effected by the extreme stress can be measured with greater precision than a small sodium change. That is, the percentage error would be larger for the determination of a small change than for a large change since the accuracy of the method is constant. Thus the large variances observed for the desiccation method and for the moderate stress (B and C, Table III) method are believed to be the result of experimental error and not physiological variation.

The "apparent volume of distribution" for potassium in most cases was calculated to be greater than 100% body weight by the immersion method and averaged about 13% body weight by the desiccation method. It thus becomes clear that the "apparent volume of distribution" has little morphological significance even when referring to a specific ion. The following sections will show that differences in values for "apparent volume of distribution" are indications that sources of ions within the crab other than the blood are participating in exchanges with the medium.

First, in Table IV it can be seen that under various aqueous osmotic stresses the blood potassium changes of the crab are approximately equal to the blood sodium changes, percentage-wise. Of the four treatments represented in Table IV only in the case where crabs are transferred from normal sea water to concentrated sea water is the ratio, sodium change (% original)/potassium change (% original) significantly less than 1.0, $P = 0.05$. The other values are not significantly different from 1.0, which means that in general, potassium and sodium are regulated approximately equally in the blood of *Pachygrapsus* when the crab is subjected to osmotic stress. It should be observed that values in Table IV are means of individual ratios, blood sodium change (% original)/blood potassium change (% original), not ratios of the mean blood changes presented in Table I. Any discrepancy between the mean of ratios and the ratio of means can be explained by the observed variances.

Now let us assume that the mean "apparent volume of distribution" for sodium in crabs exposed to moderate stresses, 38.5% body weight, represents a constant volume of fluid in the animal in which both sodium and potassium concentrations are equal to those of the blood. This particular hypothetical volume, which hereafter shall be referred to as "A–D volume," was chosen because it was determined under conditions of moderate stress and would be expected to be closer to a possible morphological space than a value obtained under conditions of extreme stress. That is, a moderate stress is closer to a normal condition than is an extreme stress.

If then a change in the quantity of an ion in the external medium of an animal were known, by knowing the concentration change in the blood and the volume, 38.5% body weight, it could be determined what fraction of an ionic exchange between crab and external medium appears in the "A–D volume." Table V, column 1 reveals that in the case of potassium less than half of a loss or gain by the medium (42% maximum) is calculated to be accounted for in the "A–D volume." This means that a tissue potassium pool (probably the intra-cellular space) is participating in the exchanges. Also in the case where the crab is exposed to extreme osmotic stresses, only part of the sodium change in the medium (84%) appears in the "A–D volume," again an indication of a salt pool. It has already been shown that the sodium exchanges which occur under extreme stress are significantly different from those occurring under moderate stress (see discussion of data in Table III). Now with respect to potassium there is no such trend with increased stress. However, the calculated ratio, change in "A–D volume"/change in medium with respect to potassium for crabs transferred from normal sea water to dilute sea water (0.22) is significantly less than 0.38, calculated for animals transferred from normal sea water to 150% sea water, $P = 0.01$, and also significantly less than 0.42, calculated for crabs treated by transferring from 150% sea water to 50% sea water, $P = 0.001$. On the other hand, 0.22 is not significantly different from the calculated value, 0.33, obtained for crabs treated by transferring from 50% sea water to 150% sea water. I have no explanation for this curious

Table V

Sodium and potassium exchanges between Pachgrapsus and ambient stress media

	Treatment		No. of crabs	(1) Calculated Change in "A-D volume" (mg.)/Change in medium (mg.)	(2) Observed Change in blood (mEq./l.)/Change in medium (mEq./l.)*	
				Mean	Mean	S.D.
A	100% sea water to 25% or 50% sea water	Na	28	0.98	2.56	0.82
		K	20	0.22	0.56	0.43
B	100% sea water to 150% sea water	Na	25	1.10	2.63	0.80
		K	24	0.38	0.998	0.70
A + B	100% sea water to dilute or concentrated sea water	Na	53	1.00**	2.60**	0.82
		K	44	0.31	0.80	0.62
C	50% sea water to 150% sea water	Na	12	0.86	2.23	0.43
		K	12	0.33	0.87	0.50
D	150% sea water to 50% sea water	Na	14	0.82	2.13	0.22
		K	14	0.42	1.08	0.35
C + D	50% or 150% sea water to 150% or 50% sea water	Na	26	0.84	2.18	0.32
		K	26	0.38	0.98	0.44

* Change in medium was caused by ions lost or gained by the crab. This change in all cases was corrected for a volume of medium equal to the weight of the crab because the ratio, weight of medium/weight of animal was not equal for all crabs.

** $$\frac{\text{"A-D volume"}}{\text{Medium volume}} \times \frac{\text{Change in blood (mEq./l.)}}{\text{Change in medium (mEq./l.)}} = \frac{\text{Change in "A-D volume" (mg.)}}{\text{Change in medium (mg.)}}$$

$$= 0.385 \times 2.60 = 1.00.$$

fact that the ratio, change in "A–D volume"/change in medium for animals transferred from 100% sea water to dilute conditions, was lower than for some of the other treatments. The important conclusions that can be made from the data contained in Table V are: a) For a given change of the respective ions in the external medium more than twice as much sodium as potassium can be accounted for in the "A–D volume," which means that a potassium source other than the blood is participating in exchanges with the medium. b) The percentage of a sodium change in the medium which can be accounted for in the "A–D volume" decreases with increased osmotic stress, again suggesting the participation of a sodium pool. c) The salt pools quantitatively have the same role in hypertonic media as in hypotonic media.

Discussion

The validity of the above conclusions concerning the presence of salt pools does not depend on the validity of the value 38.5% body weight for "A–D volume." Qualitatively, the same conclusions can be reached from the data in column 2, Table V which are the *observed* ratios, change in blood (mEq./l.)/change in medium (mEq./l.) from which the *calculated* ratios, change in "A–D volume" (mg.)/change in medium (mg.) can be derived (column 1, Table V).

The different values for "apparent volume of distribution" (Table III) cannot be interpreted as a varying morphological space filled with fluid in which sodium is dissolved in concentrations equal to those of the blood. It has been established already (Gross, 1957) that *Pachygrapsus* does not gain or lose water significantly when immersed in an osmotic stress. Therefore, a sodium pool must be contributing to the exchanges which occur between animal and medium. It will be remembered that the values for "apparent volume of distribution" for potassium are usually more than 100% body weight. This suggests, of course, that the potassium of the intra-cellular fluid, known to be in relatively high concentrations, is participating in the exchanges with the medium, in effect acting as a potassium pool.

Now by assuming a constant volume of fluid in the crab ("A–D volume") in which sodium and potassium are dissolved in concentrations equal to those of the blood, we can arrive at a quantitative estimation of the role of the salt pool for a given ion exchange between animal and medium (Table V, column 1). Figure 1 illustrates further how a salt pool might function under conditions of *extreme* stress. Here a 100-gram crab whose "A–D volume" is 38.5% body weight is immersed in 100 ml of 50% or 150% sea water. Under conditions of presumed equilibrium this results in an assumed 20% alteration in the blood sodium; *i.e.*, from 500 mEq./l. to 400 mEq./l. when in the dilute medium, and from 500 mEq./l. to 600 mEq./l. in the concentrated medium. Since blood potassium is regulated approximately equally to blood sodium percentage-wise (Table IV), the blood potassium under the above conditions will also be altered 20%; *i.e.*, from 8.0 mEq./l. to 6.4 mEq./l. in the dilute medium and 8.0 mEq./l. to 9.6 mEq./l. in the concentrated medium.

Using the volume 38.5% body weight for "A–D volume," the above concentration changes can be converted to quantities of the two ions in milligrams. Also, knowing the volume of the external medium and the concentration change there (the ratios, blood change (mEq./l.)/medium change (mEq./l.) are presented in Table V, column 2) the ion loss or gains in the medium can be expressed in milligrams. Thus it can be seen in the diagram (Fig. 1) that while 106 mg. of sodium enter or leave the "A–D volume" (solid-lined arrow), a net change of only 89 mg. occurs in the "A–D volume," that is, 84% of the flux (see Table V, column 1, C + D). The remaining 17 mg. of sodium are fixed in the salt pool in the hypertonic medium or released from the salt pool in the hypotonic medium (dotted arrows), thus significantly contributing to the mechanism of maintaining ionic and osmotic homeostasis in the body fluids.

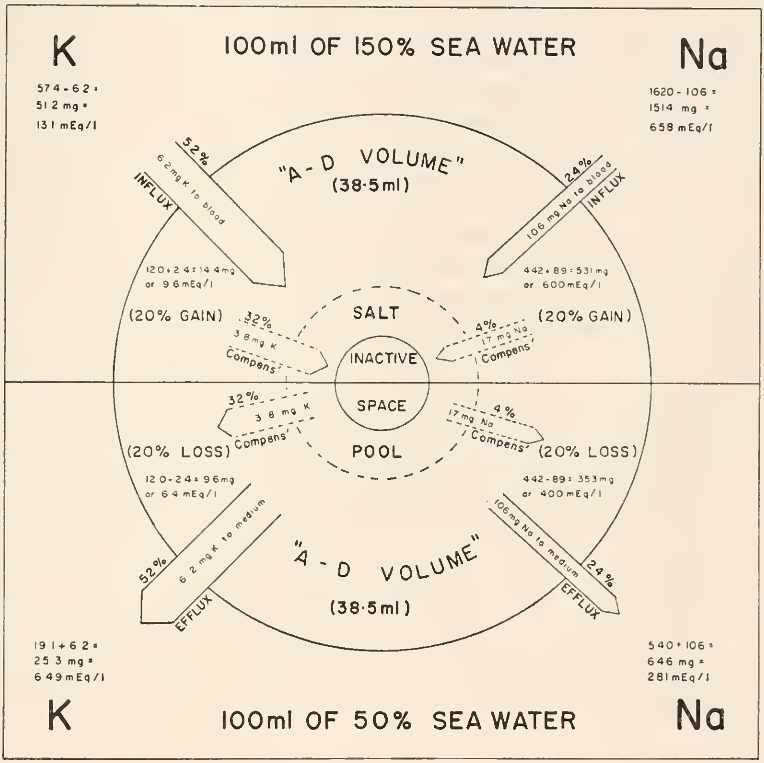

K
100ml OF 150% SEA WATER
Na
57 4 - 6 2 =
51 2 mg =
13 1 mEq/l
1620 - 106 =
1514 mg =
658 mEq/l
52%
6 2 mg K to blood
INFLUX
"A - D VOLUME"
(38·5ml)
24%
106 mg Na to blood
INFLUX
12 0 + 2 4 = 14 4mg
or 9 6 mEq/l
442 + 89 = 531mg
or 600mEq/l
(20% GAIN)
32%
3 8 mg K
Compens'
SALT
INACTIVE
SPACE
POOL
4%
17 mg Na
Compens'
(20% GAIN)
32%
3 8 mg K
Compens'
(20% LOSS)
4%
17 mg Na
Compens'
(20% LOSS)
12 0 - 2 4 = 9 6mg
or 6 4 mEq/l
442 - 89 = 353mg
or 400 mEq/l
52%
6 2 mg K to medium
EFFLUX
"A - D VOLUME"
(38·5ml)
106mg Na to medium
24%
EFFLUX
19 1 + 6 2 =
25 3 mg =
6 49 mEq/l
540 + 106 =
646 mg =
281 mEq/l
K
100ml OF 50% SEA WATER
Na

FIGURE 1. Suggested functional salt pool in a 100-gram crab immersed in osmotic stresses. Animal is represented by large circle; large square the external medium. Upper half of diagram illustrates net ion movements in 150% sea water; lower half; net ion movement in 50% sea water. Solid-line arrows indicate ion exchanges between external medium and "A–D volume" (assumed to be 38.5% body weight). Dotted arrows indicate ion exchanges between salt pool and "A–D volume." All percentages in diagram are with respect to initial blood concentrations (500mEq./l. for sodium; 8.0 mEq./l. for potassium). Numerical data are subject to small errors in rounding off. Compens' = compensation. The diagram may be read as follows: For example, for potassium loss in 50% sea water: a 100-gram crab assumed to have an "A–D volume" of 38.5% (explained in text) is found to lose 6.2 mg. of potassium to the medium, but its "A–D volume" potassium only goes down from 12.0 to 9.6 mg., a decrease of 2.4; the extra potassium is assumed to come from a pool and must be 6.2–2.4 = 3.8 mg. Therefore, the crab loses potassium equal to 52% of its initial "A–D volume" potassium, but the blood only decreases 20% and a compensation is calculated amounting to 32% of the initial potassium entering from the pool.

With respect to potassium, 6.2 mg. enter or leave the "A–D volume," but only a final change of 2.4 mg. remains in the "A–D volume," or about 38% of the flux (Table V, column 1). Again the remainder is fixed in or released from the salt pool.

It is possible that instead of compensatory exchanges occurring between salt pool and blood, salt fluxes occur directly between pool and external medium without passing into the "A–D volume." Such a phenomenon could yield the same results as presented in Table V, but it would seem negatively adaptive and also improbable because of the problem of transporting ions directly between pool and external medium through an exoskeleton which is relatively impermeable (Gross, 1957). It should be emphasized that such ion fluxes to and from the salt pool would be the same per unit blood change in 50% sea water as in 150% sea water. Passive transport from a pool containing the concentration of sodium and potassium permitting such fluxes would be extremely slow, especially through the exoskeleton. In the case of potassium, a greater net exchange occurs from the salt pool than from the "A–D volume" and presumably the blood which is separated from the external medium by tissues known to be permeable, *e.g.*, gills. While some flux of salts may occur directly between pool and medium, it seems more likely that the blood is traversed by the majority of the exchanged ions.

The events described above in Figure 1 have assumed conditions of extreme stress such as might occur by transferring an animal acclimated to 50% sea water into 150% sea water. The calculated role of the sodium pool in a lesser stress might decrease or become zero for the conditions in Figure 1 which were set up to explain the variation that occurs between two magnitudes of stress for the ratio, blood sodium change (mEq./l.)/medium sodium change (mEq./l.) (Table V, column 2). The morphological significance of "A–D volume" is obscure, but it seems possible that were the ratios in Table V, column 2, obtained under conditions of minute stress, the "apparent volume of distribution" for sodium would be smaller than 38.5% body weight and this smaller volume would have been chosen as the hypothetical constant "A–D volume." The principle would remain the same, however, namely that increased values for "apparent volume of distribution," for sodium with increased stresses does not indicate an increase in a volume of fluid, but rather participation of a sodium pool in the sodium exchanges between animal and medium.

The close agreement of values for sodium "apparent volume of distribution" obtained by the desiccation and the immersion method when animals were transferred from 50% sea water to 150% sea water or vice versa (extreme stress), is interpreted as a coincidence of values with possibly two different phenomena involved. Again, assuming the constant "A–D volume," 38.5% body weight, both the values, 45.9% body weight obtained from the extreme stress method and 48.9% body weight obtained from the desiccation method (Table III) can be explained as greater participation of sodium reservoirs. However, the value 48.9% body weight obtained by desiccation could also be explained by the participation of water pools which are inactive until conditions of desiccation exist when they are capable of replenishing water lost by evaporation. In either case, salt or water pool, the end result would be values for "apparent volume of distribution" which would be greater than those obtained under moderate immersion stresses (38.5% body weight). In both cases there would be a tendency to maintain a constancy of blood which therefore would be an adaptive end result.

The large changes in the blood potassium relative to sodium that occur during desiccation (Table II) indicate that potassium passes from some sort of intrinsic supply into the blood. The inability of *Pachygrapsus* to regulate its blood potassium under conditions of desiccation may be an important factor limiting the terrestrial behavior of this crab. Gross (1955) discusses other limiting factors with respect to land habits of *Pachygrapsus.*

The nature of the above described salt pool may be merely the formed tissues responding to an osmotic or ionic stress, thus exchanging ions from the cytoplasm or cell surface when a gradient threshold is surpassed. This interpretation possibly is corroborated qualitatively by Shaw (1955) who demonstrated that muscle fibers of the crab *Carcinus* release both sodium and potassium when the animal is immersed in dilute sea water. Also, much information is available especially concerning mammals, demonstrating that the ionic concentrations of tissues can be altered by varying the concentration of ions in the environmental fluid. For example, muscle sodium will increase in an animal perfused with hypertonic sodium chloride solution; the liver shows gains in potassium when the plasma potassium is elevated, or muscle potassium will decrease if an animal is perfused with glucose solution. Such studies and similar studies concerned with isolated tissues are reviewed by Manery (1954) and Harris (1956).

It may well be that the findings of the present investigation are manifestations of the same general cellular mechanism, illustrated by the above mentioned perfusion experiments, *i.e.*, incidental ionic changes take place in the formed tissues in response to changes in the environmental fluid. However, it should be emphasized that *Pachygrapsus,* as an aquatic animal, must contend normally not only with salts and water reaching it by way of the gut but also with the flux of salts and water which occur continuously through permeable membranes separating the body fluids and tissues from the external medium, a problem not presented to terrestrial animals. It is interesting that in *Pachygrapsus* more than twice as much potassium is estimated to exchange between external medium and the crab, than the net exchanges calculated to occur in the "A–D volume" or probably the blood, itself (Fig. 1). Yet comparing the final blood concentrations with normal blood concentrations (Table I), the blood potassium does not vary from normal more than 30%. Thus, it seems that there is a tendency to maintain the blood potassium at a constant level at the expense of the tissue or pool potassium. This suggests a method of ionic regulation in the blood without need of a special organ such as a kidney.

It is possible, then, that the above described salt pools could have a special, functional significance which would be adaptive for an aquatic animal such as a crab. Thus, normally, osmotic and ionic constancy of the blood could be maintained at least partially by salt pools which are capable of mobilizing or fixing salts to and from the blood as the situation demands. Such a device would be necessarily of temporary value only, but would be particularly advantageous for estuarine forms which could make up an osmotic deficit from their salt pools at low tide and low salinities, then replenish the pools with a minimum of work when the salinity was elevated on the in-coming tide.

These studies were aided by a contract between the Office of Naval Research, Department of the Navy and the University of California, NR 163-309.

I am pleased to acknowledge the technical assistance of Mr. Paul Holland. Also I wish to express my gratitude to Professor Theodore Holmes Bullock for his advisory assistance in the preparation of the manuscript, to Professor Timothy Prout for his advice concerning the statistical handling of the data and to Professor Ralph Smith for his critical reading of parts of the manuscript.

Summary

1. When *Pachygrapsus* is immersed in a stress medium its blood concentration is altered by a loss of ions to a hypotonic medium and a gain of ions from a hypertonic medium. Water exchanges are insignificant in magnitude.

2. The observed ratio, change in blood ions/change in medium ions yields values for "apparent volume of distribution" for the respective ions. Such values vary according to the treatment for sodium and in moderate stresses average 38.5% body weight, in extreme stresses 45.9% body weight. For potassium most values came to more than 100% body weight and do not vary with increased stress. The above ratios are the same for a hypotonic medium as for a hypertonic medium.

3. Varying values for "apparent volume of distribution" under different magnitudes of osmotic stress suggest the presence of salt pools which may represent incidental participation of the formed tissues, or may represent an adaptive mechanism which functions to assist in the ionic and osmotic regulatory mechanism.

4. In stress media blood potassium and sodium are regulated equally well, percentage-wise, but a source other than the blood participates in the exchanges of potassium between animal and medium. Thus the potassium change in the blood does not account for the total potassium change in the animal.

5. "Apparent volume of distribution" calculated from the increased blood concentration caused by a given water loss by evaporation averages 48.9% body weight for sodium and only 13% body weight for potassium. The blood potassium therefore, increases percentage-wise about four times more than blood sodium. This indicates that potassium leaves a pool (probably the intra-cellular space) to enter the blood. This appears to be a physiological failure rather than regulation, and may play a role in ecological limitations.

6. Potassium concentrations in the blood of normal crabs (*Pachygrapsus*) are less than those of sea water. When immersed in dilute sea water of lower potassium concentrations than found in the blood of normal animals, the crabs usually tend to lose potassium so that it remains less concentrated than the potassium of the medium.

LITERATURE CITED

Beadle, L. C., 1957. Comparative physiology: osmotic and ionic regulation in aquatic animals. *Ann. Rev. Physiol.*, **19**: 329–358.

Gross, W. J., 1955. Aspects of osmotic regulation in crabs showing the terrestrial habit. *Amer. Nat.*, **89**: 205–222.

Gross, W. J., 1957. An analysis of response to osmotic stress in selected decapod Crustacea. *Biol. Bull.*, **112**: 43–62.

Harris, E. J., 1956. Transport and Accumulation in Biological Systems. Academic Press Inc., New York.

HUKUDA, K., 1932. Change of weight of marine animals in dilute media. *J. Exp. Biol.,* **9**: 61–68.

JONES, L. L., 1941. Osmotic regulation in several crabs of the Pacific Coast of North America. *J. Cell. Comp. Physiol.,* **18**: 79–91.

KROGH, A., 1939. Osmotic Regulation in Aquatic Animals. Cambridge at the University Press.

MANERY, J. F., 1954. Water and electrolyte metabolism. *Physiol. Rev.,* **34**: 334–417.

PROSSER, C. L., D. W. BISHOP, F. A. BROWN, JR., T. H. JAHN AND V. WULFF, 1950. Comparative Animal Physiology. W. B. Saunders Co., Philadelphia.

PROSSER, C. L., S. W. GREEN AND T. S. CHOW, 1955. Ionic and osmotic concentrations in blood and urine of *Pachygrapsus crassipes* acclimated to different salinities. *Biol. Bull.,* **109**: 99–107.

SHAW, J., 1955. Ionic regulation in the muscle fibres of *Carcinus maenas.* II. The effect of reduced blood concentration. *J. Exp. Biol.,* **32**: 664–680.

WINKLER, A., J. ELKINTON AND A. ESENMAN, 1943. Comparison of sulfocyanate with radioactive chloride and sodium in the measurement of extra-cellular fluid. *Amer. J. Physiol.,* **139**: 239–246.

A RE-EXAMINATION OF THE OSMOTIC PROPERTIES OF THE PACIFIC HAGFISH, POLISTOTREMA STOUTI

WILLIAM N. McFARLAND AND FREDERICK W. MUNZ

Department of Zoology, University of California, Los Angeles 24, California

Investigations of various constituents of the blood of vertebrates have led to generalizations about vertebrate origins and relationships. The entirely marine myxinoids or hagfishes are morphologically primitive in the vertebrate assemblage and have unique osmotic and ionic properties. Most authors have concluded that hagfishes are approximately isotonic to their environment and that the osmotic concentration of the blood is made up largely of electrolytes. In some instances results have shown either a slight or marked hypertonicity of the blood serum with respect to the environment (summary in Table I), in contrast to the general conclusion of isotonicity.

Dekhuyzen (1904), Greene (1904) and the Schmidt-Nielsens (1923) concluded from their measurements of the freezing-point depressions of the blood sera and external medium that hagfishes must be isotonic. Smith (1932) interpreted his results, and those of Dekhuyzen and of Greene, however, to indicate a slight hypertonicity. Borei (1935), in contrast, concluded that *Myxine* was distinctly hypotonic to its environment. Krogh (1939, p. 119) considered that the blood of hagfishes is in almost complete osmotic equilibrium with sea water. In the most recent review, Black (1957, p. 187) concurs with Krogh, but her Figure 4 suggests that hagfishes are slightly hypertonic to sea water.

In view of the osmotic uniqueness of hagfishes it is important to know whether they are isotonic or are hypertonic to their environment. In the present investigation we have undertaken to determine whether the blood of the Pacific hagfish, *Polistotrema stouti,* is isotonic or hypertonic to the external medium over a range of concentrations. The chloride and sodium concentration of the blood serum and of the external environment were determined for comparison with previous work (see Table I). Since some of the variation of previous results may have been caused by different methods of handling and analysis, our own methods are described fully.

Materials and Methods

1. *Capture of animals*

On May 14, 1957, *Polistotrema stouti* (Lockington) was trapped by one of us (WNMcF) near San Diego, California. The trap was constructed from a 5-gallon can (MacGinitie and MacGinitie, 1949, p. 415), baited with two dead mackerel and set for 7 hours in 150 fathoms of water 4 miles north of North Coronado Island, Mexico. Of approximately 100 hagfish caught, 45 were transported to the laboratory at Marineland of the Pacific, Palos Verdes. California.

2. *Care of animals*

The hagfish were kept in three-gallon jars that were submerged in sea water in a large refrigerated tank (11–14° C.). Aerators were inserted through holes in the jar lids. There were no deaths under these conditions during a two-month period. Food was not offered until after the conclusion of the experiment performed on May 27. Dead mackerel were then placed in the jars on several occasions. Feeding was infrequent and the viscera only were consumed.

TABLE I

Previous measurements of osmotic pressure and chloride concentration of hagfish blood

Species	External medium		Internal medium		Internal medium[b] / External medium	Urea (mM/l.)	Source
	Δ_e[a] (° C.)	Chloride (milli-eq./l.)	Δ_i[a] (° C.)	Chloride (milli-eq./l.)			
M. glutinosa	1.73	—	1.74 1.83	—	Isotonic	—	Dekhuyzen (1904)
P. stouti	1.92	—	1.97	—	Isotonic	—	Greene (1904)
M. glutinosa	0.97 1.26 1.85 2.32	—	1.25 1.40 1.85 2.32	—	Isotonic	—	Schmidt-Nielsens (1923)
M. glutinosa	1.88	—	1.85 1.93 1.98	465 to 476	Hypertonic	2–4	Smith (1932)
P. stouti	—	384 467 530 626	—	344 414 471 570	Isotonic $Cl_i/Cl_e = 0.89$	Not detected	Bond, Cary and Hutchinson (1932)
M. glutinosa	1.90[c]	520	1.50[c]	325[d]	Hypotonic $Cl_i/Cl_e = 0.62$	58–62	Borei (1935)
M. glutinosa	—	483	—	448	Isotonic $Cl_i/Cl_e = 0.93$	—	Cole (1940)[e]
M. glutinosa	—	592[f]	—	576[f]	Isotonic $Cl_i/Cl_e = 0.91$[f]	2–4	Robertson (1954)

[a] Subscript e = external; i = internal.

[b] Interpretation of original author.

[c] Calculated from chloride and urea concentrations.

[d] Analysis of whole blood; low value may be related to low chloride concentration of red blood cells (see Robertson, 1954).

[e] Data of Smith, obtained in 1927.

[f] Chloride values are millieq./kg. water; ratio has been converted so that it is directly comparable to the others.

3. *Adjustment of animals to sea water of three different concentrations*

In addition to natural sea water (100%) a dilute medium of approximately 85% sea water was obtained by mixing 100% sea water and distilled water. Sea water was concentrated by boiling to half the original volume. This was mixed with natural sea water to provide a medium of approximately 115% sea water. The precise concentrations were obtained from freezing-point measurements. Hagfish were transferred in the three-gallon jars directly to sea water of these three concentrations. After an adjustment period of 30 hours, in which temperature was controlled by partially immersing the jars in the refrigerated tank, the hagfish were transported in these vessels to the University of California, Los Angeles, where the sampling was performed. Upon arrival the jars were placed in a cold room (2° C.) for a period of 1–3 hours. During this time the water temperature declined from 11.0° C. to a low value of 6.5° C. These temperatures are within the normal temperature range hagfish encounter.

4. *Blood sampling*

To facilitate withdrawal of blood samples the hagfish were stretched on a board with hemostats. Blood was removed in a syringe from the subcutaneous sinus. The freezing point of the first 0.2 ml. of whole blood was determined while more blood was withdrawn from the animal. Difficulty in obtaining blood from the thoracic or caudal hearts or from the systemic blood vessels made use of the sinus necessary.

The subcutaneous sinus of hagfishes is part of the blood circulatory system and not part of the lymphatic system (Cole, 1926, p. 322). It is one of a large system of sinuses which in cyclostomes appear to partially replace capillary beds. Blood flows into this extensive sinus from arteries of the snout and slowly moves posteriorly to the tip of the tail, where it is collected in a special vessel and pumped into the systemic circulation by the caudal hearts.

After withdrawal blood was placed in a 12-ml. centrifuge tube. A preliminary trial had shown that centrifugation of uncovered blood samples concentrated the blood sera by evaporation. The blood samples were therefore covered with paraffin oil immediately after withdrawal. No anticoagulant was necessary. Following centrifugation at 3500 times gravity for 20 minutes to remove cellular elements, the yellow or reddish supernatant fluid was drawn from beneath the paraffin oil in a slender-tipped pipette and placed in another centrifuge tube. Samples for chloride and sodium ion determination were removed and again a paraffin oil cover was added. The blood sampling and whole-blood freezing-point determinations were performed at the same time; freezing points of the serum were measured within the next 24 hours. The covered serum samples were stored in a refrigerator at 5° C.

The total length, weight and sex were determined for each specimen following blood removal. Specimens ranged in total length from 300 mm. to 460 mm. Their weights varied from 41.4 gm. to 135.3 gm.

5. *Freezing-point determinations*

Freezing points were measured with the Fiske Osmometer, which employs a thermistor. Samples of 0.2 ml. were placed in the small sample adapter of this instrument and rapidly supercooled in the propylene glycol bath (– 10° C.). Vibration of a wire initiated freezing of the sample; resistance of the thermistor element, which was located in the freezing mixture, was balanced with a variable resistance. The osmometer had been previously calibrated with standard NaCl solutions; individual determinations of single samples varied no more than ± 0.02° C. Just before withdrawal of the hagfish from each container, freezing points of a water sample from that container were determined three times and the mean of these values recorded. Whole-blood and serum samples were determined only once to prevent denaturation of the protein fraction from changing the osmotic pressure of the frozen and thawed samples.

6. *Chloride and sodium analysis*

The chloride concentrations of the medium and serum were determined by a modification of the Volhard method reported by Keys (1937) and Consolazio, Johnson and Marek (1951). The small volume of blood obtained did not allow replicate determinations; each result therefore represents one titration. A mean error of 3.3% was obtained in analyses of standard NaCl soluitons. Chloride values are reported in milliequivalents per liter. Sodium was determined with flame photometry by the direct method. A Beckman Model B spectrophotometer with flame attachment was used in this analysis. A standard curve was established from a solution containing sodium, potassium, calcium and magnesium in the proportions reported for these elements in sea water of 19 parts per thousand chlorinity (Sverdrup, Johnson and Fleming, 1942, p. 186). No special provision was made to remove proteins prior to analysis, nor was any agent, such as isopropyl alcohol, used in the diluting solution to aid in ignition. The mean error determined was 3.2%.

RESULTS

Osmotic data

Experiment 1

Jars of 85.5%, 100%, and 116.1% sea water (percentages based on freezing-point determinations), each containing three hagfish which had been kept at these concentrations for 30 hours, were brought to the laboratory on May 20, 1957. Freezing-point depressions of the external medium and of whole blood and serum samples of each hagfish were measured (Table II). Initially blood was drawn from one animal at each concentration (specimens 1D, 1N and 1C, Table II) and the osmotic pressure determined. The second animals tested at each sea water concentration (2D, 2N and 2C) had higher osmotic pressures. One hour was allowed to pass before specimens 3D, 3N, and 3C were removed and the blood samples obtained. The osmotic pressure of these last three hagfish returned to values near the concentrations of their respective environments.

This experiment indicated that *Polistotrema stouti* becomes isotonic or slightly hypertonic to its environment over the range of salinities from $\Delta = 1.59$ to 2.16° C. The Δ's of serum and whole blood were almost the same (ratio of Δ_s/Δ_{wb} from 99.4% to 102.3%). The slight hypertonicity of whole blood and serum to the external medium appeared to increase during the experiment, but after an hour the blood returned toward isotonicity with the environment. These changes were quite uniform throughout the range of salinities tested.

Experiment 2

A second experiment was performed on May 27 with sea water at 4 concentrations from 83.5% ($\Delta = 1.57°$ C.) to 116.0% ($\Delta = 2.18°$ C.). Only a single hagfish was kept in each jar. The freezing point of the sea water in each container was measured just before removal of the hagfish.

TABLE II

Freezing-point depressions of whole blood (Δ_{wb}) and serum (Δ_s) of hagfish which had been kept in 3 different concentrations (Δ_e) of sea water for 30 hours. D, N, and C refer to dilute (85%), normal (100%) and concentrated (116%) media

	Sea water concentration (%)	Δ_e (° C.)	Animal number	Δ_{wb} (° C.)	Δ_s (° C.)	$\frac{\Delta_{wb}}{\Delta_e}$ (%)	$\frac{\Delta_s}{\Delta_e}$ (%)	$\frac{\Delta_s}{\Delta_{wb}}$ (%)
Experiment 1	85.5	1.59	1D	1.59	1.58	100.0	99.4	99.4
			2D	1.64	1.65	103.1	103.8	100.6
			3D[a]	1.61	1.60	101.3	100.6	99.4
	100.0	1.86	1N	1.87	1.89	100.5	101.6	101.1
			2N	1.93	1.94	103.8	104.3	100.5
			3N[a]	1.88	1.89	101.1	101.6	100.5
	116.1	2.16	1C	2.21	2.21	102.3	102.3	100.0
			2C	2.22	2.27	102.8	105.1	102.3
			3C[a]	2.19	2.23	101.4	103.2	101.8
Experiment 2[b]	84.6	1.59	4D	1.59	1.59	100.0	100.0	100.0
	83.5	1.57	5D	1.58	1.57	100.6	100.0	99.4
	100.0	1.88	4N	1.89	1.90	100.5	101.1	100.5
	100.0	1.88	5N	1.91	1.88	101.6	100.0	98.4
	105.9	1.99	4C	1.96	2.00	98.5	100.5	102.0
	116.0	2.18	5C	2.19	2.22	100.5	101.8	101.4

[a] A one-hour wait intervened before blood samples were drawn from the last animal in each container.

[b] Each hagfish kept in separate container.

The results (Table II) leave no doubt that the hagfish under these conditions were isotonic, not hypertonic, to the external medium (Δ_e between 1.57 and 2.18° C.). The Δ of 1.99° C. in one of the jars of concentrated sea water was the result of an unintentional dilution with 100% sea water during the preliminary adjustment period. The individual at this concentration, however, matched its osmotic environment about as well as the others.

In both experiments animals at different concentrations showed perceptible differences in the available volume and the apparent viscosity of blood. Those in diluted sea water provided a greater quantity of more "watery" blood than normal.

In concentrated sea water smaller amounts of more viscous blood were obtained. The external appearance of the animals did not differ appreciably in the three sea water concentrations.

Ionic data

The serum chloride and sodium concentrations corresponding to the freezing points reported in Experiments 1 and 2 are listed in Table III. Like the freezing-point values, the serum chlorides of the hagfish in Experiment 1 also show an increase in concentration in the second animals tested (specimens 2D, 2N, and 2C) at each concentration. One hour later the hagfish (3D, 3N, 3C) showed a decline in serum chloride toward or below the original value. Where data were obtained, the sodium concentrations of sera in Experiment 1 also exhibit this trend.

TABLE III

Sodium and chloride concentrations of the serum of Polistotrema stouti in Experiments 1 and 2

	Animal number	Total concentration[a] (milliosmols)		Milliequivalents/liter			
		Medium	Blood serum	Cl_e	Cl_i	Na_e	Na_i
Experiment 1	1D	853	849	441	419	330	—
	2D	853	886	441	431	330	360
	3D	853	861	441	385	330	335
	1N	999	1018	514	490	480	450
	2N	999	1043	514	516	—	—
	3N	999	1015	514	487	480	405
	1C	1162	1187	619	549	590	520
	2C	1162	1220	619	538	590	520
	3C	1162	1200	619	472	—	—
Experiment 2	4D	855	855	449	370	360	415
	5D	847	845	450	435	—	—
	4N	1010	1023	469	431	390	430
	5N	1010	1012	480	492	—	—
	4C[b]	1071	1077	530	502	405	455
	5C	1172	1193	576	529	570	475

[a] Determined from freezing-point depression.

[b] 106% sea water.

DISCUSSION

From the experiments described above it is clear that the Pacific hagfish, *Polistotrema stouti*, is isotonic to the external medium, given time (30 hours in these experiments) to adjust. A slight apparent hypertonicity (no more than 5%) which developed during the course of Experiment 1 was eliminated in Experiment 2 by keeping the hagfish separate. In Experiment 2 the average of $\Delta_{wb}/\Delta_e = 100.3\%$ and of $\Delta_s/\Delta_e = 100.7\%$. These values indicate complete isotonicity, within experimental error of the methods used.

Comparison of Experiments 1 and 2 offers a possible explanation for some of the differences reported by earlier workers. In handling the hagfish prior to withdrawal of blood it was impossible to prevent them from secreting slime copiously. In Experiment 1 removal of the first animal from each jar disturbed the others and caused sliming. The blood of the second animal from each medium was more concentrated than the first, but after an hour's wait the third animal from each medium was nearly isotonic. It seemed possible, therefore, that sliming induced a temporary hypertonicity of the blood. Support for this hypothesis was obtained in a subsequent experiment, in which as many as four small blood samples were withdrawn from single individuals at different times. Initial blood samples indicated isotonicity with sea water; production of slime was followed by hypertonicity (freezing-point measurements of whole blood samples) of 1–3%, which lasted an hour or more. Much greater increases in the blood concentration were induced by rough handling or by placing the hagfish in a dry atmosphere. After a combination of these treatments for 10 minutes, the blood became 15–25% hypertonic to sea water. Osmotic recovery was nearly complete within 24 hours. The slight hypertonicity of hagfish blood indicated in the results of Dekhuyzen (1904), Greene (1904) and Smith (1932) may have been caused by handling or the secretion of slime. This could not account for the hypotonic values calculated by Borei (1935).

TABLE IV

The contribution of sodium and chloride ions to the osmotic pressure of Polistotrema stouti. Ratios are mean values computed from the data of Table III. Parentheses denote the number of determinations from which each mean was computed

Concentration of medium (%)	Cl_i/Cl_e	Na_i/Na_e	$\frac{Na_e + Cl_e}{\text{milliosmols}_e}$[a]	$\frac{Na_i + Cl_i}{\text{milliosmols}_i}$[a]
85	0.918(5)	1.088(3)	0.914(4)	0.882(3)
100	0.970(5)	0.952(3)	0.946(3)	0.881(3)
116	0.874(5)	0.914(4)	0.985(4)	0.874(4)
Grand mean	0.918(15)	0.965(10)	0.953(11)	0.878(10)

[a] Determined from freezing-point depression.

Under conditions of the present experiments hagfish become isotonic to 84–116% sea water ($\Delta_e = 1.57$ to $2.18°$ C.). The limits of their ability to endure changed external concentrations were not tested and it is not clear whether they might be able to regulate their osmotic concentration in some range other than 84–116% sea water. An insufficient number of hagfish was obtained to investigate this problem; work will be continued as more animals become available. The mean value (15 animals) of Cl_i/Cl_e was 0.92, which is similar to reports of most other authors (see Table I). In Robertson's paper (1954) on *Myxine* the sum of Cl_i and Na_i values accounted for 95.9% of the total osmotic concentration of the blood. In the present work this ratio was 95.3%, showing the close agreement of our results with his. For computation of this ratio the grand mean of $Na_i + Cl_i$/milliosmols$_i$ (Table IV) was divided by 0.921, the factor given by Robertson to convert molar to molal concentrations in *Myxine*.

About 90% of the total osmotic concentration of the serum is due to sodium and chloride ions (Table IV); Robertson (1954) showed that other ionic constituents account for most of the remaining concentration. Both he and Smith (1932) found that there were 2–4 mM/l. of urea in the blood of *Myxine*. The high values of 58–62 mM/l. obtained by Borei (1935) are probably incorrect (non-specific tests were performed on whole blood samples; see Robertson, 1954). In a personal communication Dr. Ernest Baldwin informs us that he has not been able to detect urea in the liver of *Polistotrema*. He points out that there is little likelihood that it would occur elsewhere in the body when absent from the liver. Dr. Baldwin, like Black (1957, p. 182), suggests that the nature of the food would affect the urea content of the tissues. It is possible that even the very low concentrations of urea reported by Robertson and Smith had an exogenous origin. Urea cannot have an importance in the osmotic composition of hagfishes comparable to that which it has in elasmobranchs.

Although the hagfishes resemble other vertebrates in the ratios of the ionic constituents of their blood (Cole, 1940; Robertson, 1954), they are unique among vertebrates in being isotonic to sea water and having the osmotic concentration of the blood composed almost entirely of ionic constituents. Hagfishes are poikilosmotic within the range of sea water concentrations investigated, which encompass those of the marine habitats they normally occupy.

We wish to acknowledge the assistance of Drs. David Jensen and Arthur Kelly in obtaining the specimens, as well as the University of California at La Jolla for use of the vessel *T-441*. Dr. Ernest Baldwin has kindly allowed us to use an unpublished chemical analysis of hagfish liver. Mr. Harry Hansen of the Long Beach Water Treatment Plant made available a flame photometer and assisted in the sodium analyses. To Drs. Boyd W. Walker and Frederick Crescitelli go our thanks for their interest and suggestions during the investigation. Mr. Richard Rosenblatt read the manuscript critically.

SUMMARY

1. Determinations of the osmotic pressure of whole blood and serum show that *Polistotrema stouti* is isotonic in sea water concentrations from 85 to 116%.

2. Hypertonicity of the blood can be induced experimentally by disturbance of the animals. This factor could account for hypertonic values reported previously.

3. Serum sodium and chloride account for 88% of the total osmotic pressure. The mean Cl_i/Cl_e ratio equals 0.92. The hypertonicity which can be produced by disturbance is reflected by a rise in the serum sodium and chloride concentrations.

4. Urea is absent from the liver and is considered to have no significance in the osmotic composition.

LITERATURE CITED

BLACK, V. S., 1957. Excretion and omsoregulation. *In:* Brown, M. E., The Physiology of Fishes. Vol. 1. Academic Press, New York.

Bond, R. M., M. K. Cary and G. E. Hutchinson, 1932. A note on the blood of the hag-fish *Polistotrema stouti* (Lockington). *J. Exp. Biol.,* **9**: 12–14.

Borei, H., 1935. Über die Zusammensetzung der Körperflussigkeiten von *Myxine glutinosa* L. *Arkiv för Zoologi,* **28B**: 1–5.

Cole, F. J., 1926. A monograph on the general morphology of the myxinoid fishes, based on a study of *Myxine*. Part 6. The morphology of the vascular system. *Trans. Roy. Soc. Edinburg,* **54**: 309–342.

Cole, W. H., 1940. The composition of fluids and sera of some marine animals and of the sea water in which they live. *J. Gen. Physiol.,* **23**: 575–584.

Consolazio, C. F., R. E. Johnson and E. Marek, 1951. Metabolic Methods. C. V. Mosby Co., St. Louis.

Dekhuyzen, M. C., 1904. Ergebnisse von osmotischen Studien, namentlich bei Knochenfischen, and der biologischen Station des Bergenser Museums. *Bergens Museums Aarbog,* **1904** (8): 3–7.

Greene, C. W., 1904. Physiological studies of the chinook salmon. *Bull. U. S. Bureau of Fisheries,* **24**: 431–456.

Keys, A., 1937. The microdetermination of chlorides in biological materials. *J. Biol. Chem.,* **119**: 389–403.

Krogh, A., 1939. Osmotic Regulation in Aquatic Animals. Cambridge at the University Press.

MacGinitie, G. E., and N. MacGinitie, 1949. Natural History of Marine Animals. McGraw-Hill Book Co., New York.

Robertson, J. D., 1954. The chemical composition of the blood of some aquatic chordates, including members of the Tunicata, Cyclostomata and Osteichthyes. *J. Exp. Biol.,* **31**: 424–442.

Schmidt-Nielsen, S., and S. Schmidt-Nielsen, 1923. Beitrage zur Kenntnis des osmotischen Druckes der Fische. *Det kongelige norske Videnskabers Selskabs Skrifter 1923–1924,* **No. 1**: 1–24.

Smith, H. W., 1932. Water regulation and its evolution in the fishes. *Quart. Rev. Biol.,* **7**: 1–26.

Sverdrup, H. U., M. W. Johnson and R. H. Fleming, 1942. The Oceans. Their Physics, Chemistry, and General Biology. Prentice-Hall, New York.

THE ACOUSTICAL BEHAVIOR OF SOME FISHES IN THE BIMINI AREA[1, 2]

JAMES M. MOULTON

Bowdoin College, Brunswick, Maine

While many kinds of marine organisms are recognized as sound sources, it is not clear in most cases under what conditions the species concerned became sonic in nature, nor what patterns of behavior the sounds normally accompany. Spontaneous sound production, by many fishes known to produce sounds, tends to become partially or completely suppressed in captivity (Dobrin, 1947; Fish, 1948; Moulton, 1956b); one must frequently resort to artificial stimuli such as handling or electric shock (Fish, 1954; Fish *et al.*, 1952) to elicit any sound for study. Failure of free fishes to respond consistently to man-made sounds introduced into the water (Moulton and Backus, 1955) has aroused considerable interest, evident in writings since the time of Aristotle, in the role that the sounds of fishes themselves play in nature.

In order to obtain further information on the production of fish sounds and their significance to fish behavior, the period from 13 June to 13 August 1956 was spent at the Lerner Marine Laboratory of the American Museum of Natural History on North Bimini, Bahama Islands. A glass-panelled power boat and the clarity of the water facilitated extensive observation of near-shore fishes during underwater listening, and well-stocked fish pens provided opportunity for close observation of such fishes in a reasonably natural environment. Recordings of marine sounds, accompanied by notes on simultaneous observations, have been compared with recordings made in the laboratory from carefully identified specimens. The identity of certain sounds recorded at sea and in the laboratory has been determined by this aural comparison and by the study of vibragrams made at the Woods Hole Oceanographic Institution.

The fishes most frequently heard along the western edge of the Great Bahama Bank in the Bimini area, the Nassau grouper, *Epinephalus striatus* (Bloch), and the squirrelfish, *Holocentrus ascensionis* (Osbeck), are inhabitants of relatively shallow water. That sightings of the Nassau grouper and squirrelfish occurred only in areas where incidence of their calling was high, and that these species were generally observed in areas where their sounds were recorded, indicates a value of underwater listening in studying the distributions, even limited ones, of some sound-producing species.

Listening equipment used in the investigation consisted of an AX-58-C Rochelle salt hydrophone and a Woods Hole Suitcase amplifier; an undesignated Rochelle

---

[1] Contribution No. 957 from the Woods Hole Oceanographic Institution.

[2] The work was performed at the Lerner Marine Laboratory of the American Museum of Natural History and at the Woods Hole Oceanographic Institution, under grants of the Institution and of the Bowdoin College Faculty Research Fund established by the Class of 1928. Preparation for publication has been facilitated by Research Grant NSF-G4403 of the National Science Foundation.

Table I

Fishes making no sounds or only chewing sounds during the Bimini study

Family	Species	Comment
Acanthyridae	*Acanthus coeruleus*	1 specimen
Antennariidae	*Histrio histrio*	1 specimen
Apogonidae	*Apogon maculatus*	1 specimen
Balistidae	*Canthidermis sabaco*	Several individuals
Belonidae	*Strongylura marinus*	1 specimen
Chaetodontidae	*Chaetodon striatus*	1 specimen
	Pomacanthus paru	A 4″ specimen
Clupeidae	*Jenkinsia lamprotaenia*	Large school and individual specimens
Echeneididae	*Phtheirichthyes lineatus*	2 specimens
Fierasferidae	*Carapus affinis*	1 specimen
Gerridae	Unidentified majarra	Several specimens
Gobiidae	*Bathygobius soporator*	Feeding sounds recorded from a group. Male known to call in the breeding season (Tavolga, 1956).
Haemulidae	*Haemulon album*	Several specimens
	Haemulon parra	Several specimens
Labridae	*Halichoeres radiatus*	Feeding sounds recorded
Lutianidae	*Lutianus griseus*	1 specimen
Malacanthidae	*Malacanthus plumeri*	1 specimen
Mullidae	*Upeneus maculatus*	Feeding sounds recorded
Ogocephalidae	*Ogocephalus radiatus*	1 specimen
	Ogcocephalus vespertilio	1 specimen
Orectolobidae	*Ginglymostoma cirratum*	1 specimen
Pristidae	*Pristis pectinatus*	1 specimen
Rachycentridae	*Rachycentron canadus*	1 specimen
Scorpaenidae	*Scorpaena plumeri*	1 specimen
Serranidae	*Promicrops itaiara*	1 specimen
Sparidae	*Calamus providens*	1 specimen
Sphyraenidae	*Sphyraena barracuda*	1 specimen

salt hydrophone and a modified Heathkit amplifier Model A-7C, or an Ekotape microphone Model 205. Recordings were made at 7½ and 3¾ in./sec. on the Ekotape tape recorder and selected recordings have been analyzed on a vibration frequency analyzer, the Kay Vibralyzer. Sound-generating equipment used during the investigation consisted of a Hewlett-Packard audio oscillator Model LAJ or the Ekotape tape recorder, a Craftsman C550 amplifier, and a QBG transducer. All observations at sea were made in calm weather from the 30-foot motor launch RESEARCH of the Lerner Marine Laboratory, between 0800 and 1400 hours from 9 July to 12 August. Suitcase amplifier settings varied from 0 + 14 − 20 to 0 + 10 − 5. During work at sea, the hydrophone was suspended just above the bottom, or so that it cleared the bottom in the shoalest water in the listening area at drift stations.

Forty species of fishes, distributed among 29 families, were studied at Bimini

(Tables I and II). The stimuli used in eliciting sound in the laboratory were those of handling, capture in a net or feeding, or a combination of these. All sounds reported were recorded from isolated, submerged specimens, except the pectoral fin drumming and tooth stridulation of individual triggerfishes (Balistidae) which were not heard while the fish were being handled under water. All other sounds described were produced in air as well as under water, except the snap of the demoiselle, *Pomacentrus leucosticus* (Mueller and Troschel), which was recorded only from submerged specimens.

The species which produced no detectable sounds other than those of chewing

TABLE II

Characteristics of fish sounds recorded at Bimini

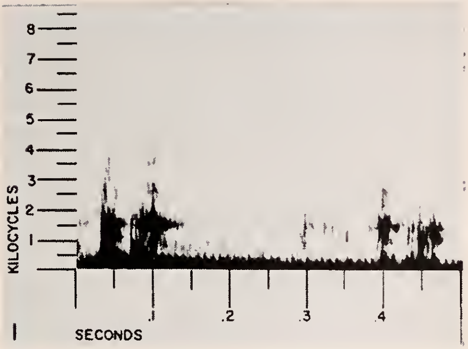
Family	Species	Vibration frequency analysis
Balistidae	*Balistes vetula* in air at microphone	Sound: Toothplate stridulation Duration: .12 sec./stridulation Frequency span: 0 to above 8 kc. Predominant intensities: .7–1.8 kc., 2.1–3.8 kc.
	Melichthys piceus in air at microphone	Sound: Toothplate stridulation Duration: .06 to .1 sec./stridulation Freq. span: 0 to above 8 kc. Pred. int.: 1.2 to 2.3 kc.
	B. vetula in air at microphone	Sound: Pectoral fin-drumming Duration: .03 sec./pulse Freq. span: 0 to 5.8 kc. Pred. int.: 1 to 1.7 kc.
	M. piceus in air at microphone	Sound: Pectoral fin-drumming Duration: .02 to .04 sec./pulse Freq. span: 0 to above 8 kc. Pred. int.: .7 to 2.2 kc.
	Feeding of a mixed group of the two spp. at AX-58-C hydrophone	Freq. span: 0 to above 8 kc. Pred. int.; .6 to 2.9 kc.
Carangidae	*Caranx hippos* hand-held in aquarium at hydrophone	Sound: Stridulation, pharyngeal teeth Duration: .06 sec./stridulation Freq. span: 0 to above 8 kc. Pred. int.: .3 to 1.2 kc., 1.7 to 3.3 kc.
Chaetodontidae	*Angelichthyes ciliaris* in cement tank at AX-58-C hydrophone	Sound: Grunt, single or repeated Duration: .06 to .1 sec./grunt Freq. span: 0 to 1.1 kc. Pred. int.: Below .5 kc.
	Pomacanthus arcuatus W. of Turtle Rocks at AX-58-C hydrophone	Sound: Grunt or moan-like sound Duration: .04 to .2 sec. Freq. span: 0 to 1.5 kc. Pred. int.: Below .5 kc.

TABLE II—*Continued*

Family	Species	Vibration frequency analysis
Diodontidae	*Diodon hystrix* hand-held in aquarium at hydrophone	Sound: Toothplate stridulation Duration: .09 sec./stridulation Freq. span: 0 to 8 kc. Pred. int.: 2.5 to 5.5 kc., with narrow intensity bands at 3, 4.3 and 5 kc.
Haemulidae	*Haemulon sciurus* hand-held in aquarium at hydrophone	Sound: Stridulation, pharyngeal teeth Duration: .02 to .1 sec./stridulation Freq. span: 0 to above 8 kc. Pred. int.: 1.5 to 4 kc.
Holocentridae	*Holocentrus ascensionis* in aquarium (Fig. 10) and at Turtle Rocks (Fig. 12); AX-58-C hydrophone	Sound: Thump-like single, or volleyed Duration: .04 to .1 sec./thump Freq. span: 0 to above 4 kc. Pred. int.: Below 1 kc.
Pomacentridae	*Pomacentrus leucostictus* in aquarium; male pursuing others of species at hydrophone	Sound: Single or repeated snaps Duration: .02 sec./snap Freq. span: 0 to 1.5 kc. Pred. int.: 0 to .8 kc.
Serranidae	*Epinephalus adsencionis* in cement tank at AX-58-C hydrophone	Sound: Vibrant grunt, single or repeated Duration: .04 to .1 sec./grunt Freq. span: 0 to .9 kc. Pred. int.: 0 to .3 kc.
	Epinephalus striatus in live car at AX-58-C hydrophone	Sound: Vibrant grunt, single or repeated Duration: .1 to .2 sec./grunt Freq. span: 0 to 2 kc. Pred. int.: 0 to .4 kc.
Tetradontidae	*Spheroides spengleri* hand-held in aquarium at hydrophone	Sound: Toothplate stridulation Duration: .07 sec./stridulation Freq. span: 0 to above 8 kc. Pred. int.: 1.3 to 3 kc.

are shown in Table I. Twelve species producing other-than-chewing sounds are listed in Table II, together with data derived from vibration frequency analysis. Of this latter group, only three species were identified as sources of sounds recorded during listening at sea—squirrelfish, Nassau grouper and black angelfish, *Pomacanthus arcuatus* (Linnaeus), all of which use the air bladder in sound production.

No individual free fishes were identified at sea as sources of stridulatory noises. Vibration frequency analysis of these latter sounds from recordings made at sea is rendered somewhat difficult by a broad band of sound with predominant intensities between 2 and 6 kc., which is characteristic of warmer seas, and which has generally been ascribed to snapping shrimp. It is not unlikely, however, that sounds created by the teeth of reef fishes (Table II—feeding of balistids) may contribute to this sound band, which on vibration frequency analysis tends to obscure upper frequency ranges of various sounds clearly discernible below the 2-kc level.



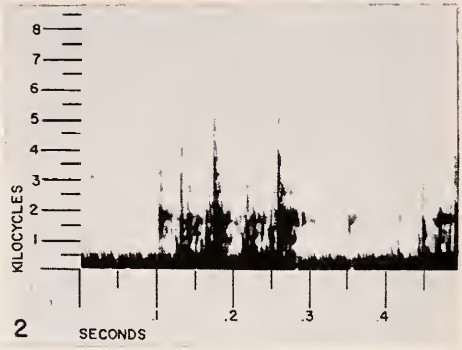
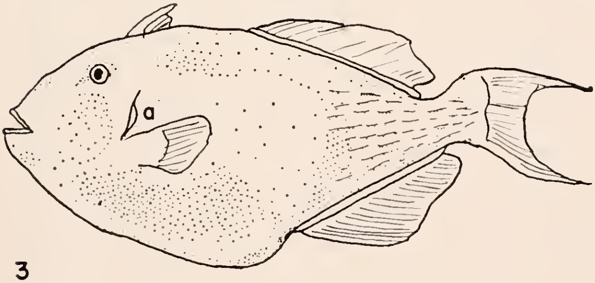


FIGURE 1. The pectoral fin-drum of *Balistes vetula* in air.
FIGURE 2. The pectoral fin-drum of *Melichthys piceus* in air.

SOUND PRODUCTION AND BEHAVIOR OF BIMINI FISHES

In the following account, each family of which sound-producing representatives were studied at Bimini, is dealt with from the points of view of the sounds produced and the correlated behavior. Initial references are to earlier descriptions dealing with sound production in the families concerned.

Balistidae (Bridge, 1910, pp. 357, 361; Fish, 1948, pp. 15–19, 1954, pp. 62–65; Schultz and Stern, 1948, p. 132). The queen triggerfish, *Balistes vetula* L., and the black triggerfish, *Melichthys piceus* (Poey), each possesses above the base of the pectoral fin a thin membrane lying lateral to the air bladder and covered by scales larger and more plate-like than those elsewhere on the body (Figs. 3, 4), a characteristic of these genera of the Balistidae (Evermann and Marsh, 1900). Males and females removed from the water and handled, frequently elevated and rapidly fluttered the pectoral fins against this region, as described by Schultz and Stern (1948), resulting in the production of a throbbing sound (Table II; Figs. 1, 2). Bridge (1910, p. 357) attributes a throbbing sound primarily to movements of the pectoral girdle.



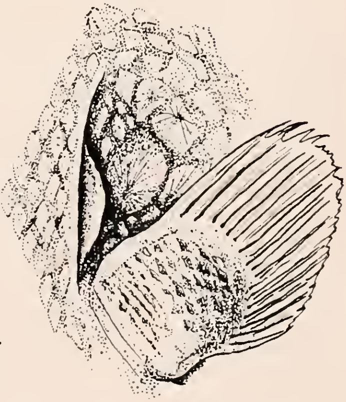
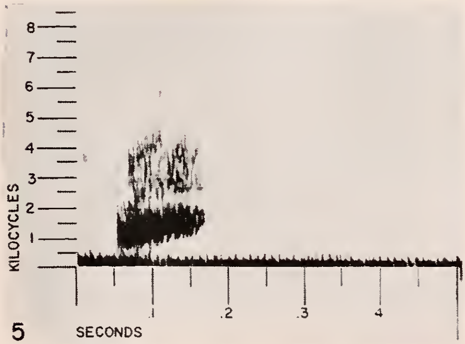


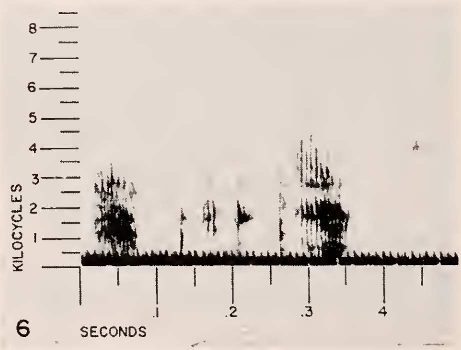
FIGURE 3. Outline of *M. piceus* showing (a) position of drumming membrane posterior to the gill opening. ×3.
FIGURE 4. Detail of the drumming membrane of *M. piceus*. ×2.5.

Differentiation of the "drumming membrane" is not apparent externally in the ocean triggerfish (*Canthidermis sabaco* Poey), nor does this species during handling move the pectoral fins to the drumming position. Similarly, the toothplate stridulation readily demonstrated by the queen and black triggerfishes (Table II; Figs. 5, 6) during handling out of water was not performed by the several ocean triggerfish studied.

While tooth stridulation and pectoral fin drumming were not heard from isolated triggerfishes handled underwater, recordings made during the feeding about the hydrophone of a captive population of queen and black triggerfishes showed predominant intensities of accompanying sounds between .6 and 2.9 kc. (Table II), which essentially spans the frequency ranges of predominant intensities obtained during tooth stridulation by these species in air. It was not possible to ascribe any specific sounds recorded at sea to triggerfishes, although their feeding activity probably contributed to background sounds recorded. They are common in the area studied.



5



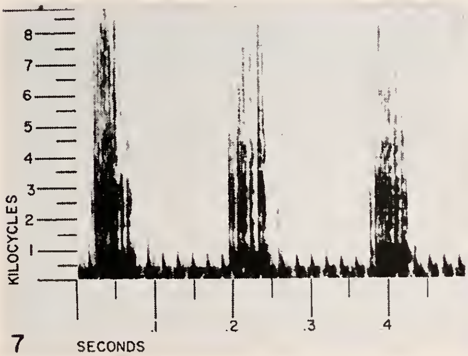
6

FIGURE 5. Tooth stridulation of *B. vetula* in air.
FIGURE 6. Tooth stridulation of *M. piceus* in air.

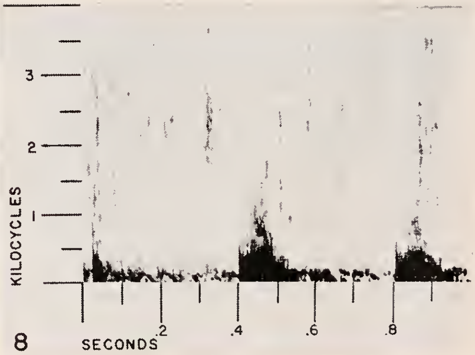
Carangidae (Bridge, 1910, p. 363; Fish, 1948, pp. 25–30). The pharyngeal tooth stridulation of *Caranx hippos* (Linnaeus) was recorded in a laboratory aquarium during handling of a 3.5-inch individual (Table II; Fig. 7). The sound recorded was not identified in any recording made at sea. Similar sound production in a related species, *Caranx crysos* (Mitchill), has been described by Fish (1954), but the thump she describes as occurring during shock was not heard during handling of the small specimen, nor was any detectable sound recorded from adult carangids swimming around and past the hydrophone at sea. A local fisherman related the stridulatory sound to "rattling of the ear bones" and asserted that a hooked specimen making this noise attracts other individuals of the species.

Chaetodontidae (Bridge, 1910, p. 361). The vibrant deep grunts of the queen, *Angelichthyes ciliaris* (Linnaeus), and black, *Pomacanthus arcuatus* (L.), angelfishes (Table II) are not easily distinguished from those of the serranids with which they may occur. Vibration frequency analysis shows (Fig. 8) a tendency for highest frequencies of the angelfish grunts to be located in the middle of the call, whereas serranid grunts tend to be initiated with high frequency spikes. Each may vary in the direction of the other, however, and since serranids and angelfishes tend to occur in similar areas, the sounds of the two may be confused. Field and laboratory observations indicated that serranids are far more prolific in call production under ordinary circumstances than are the angelfishes.

Under laboratory conditions, both queen and black angelfishes produce the grunt during feeding on bits of conch, and when startled to quickened swimming by an observer. The deeply recessed air bladder, as seen in the black angelfish, bears no intrinsic muscles, and sound production is due to the contraction of axial musculature adjacent to the air bladder. Each quick motion of a black angelfish nibbling at the hydrophone at sea resulted in a brief grunt, although more leisurely swimming of both species in laboratory tanks was not accompanied by sound production. Handling of black angelfish under water brings forth brief grunts of low intensity, coinciding with body muscle contractions. Uniquely among species studied, the black angelfish, usually in pairs, readily approached and butted against the hydrophone at sea.



7



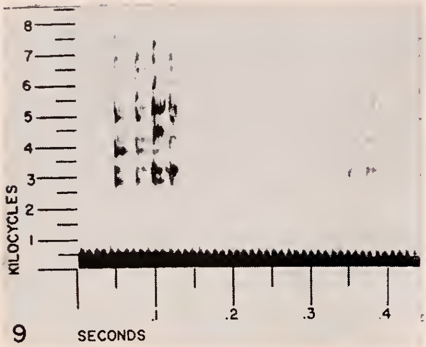
8

FIGURE 7. Pharyngeal tooth-stridulation of *Caranx hippos* in aquarium.

FIGURE 8. A short and two longer calls of *Pomacanthus arcuatus* west of Turtle Rocks, the latter during approach of another *P. arcuatus.* Snapping shrimp in background.

The deeply recessed position of the air bladder brings it into intimate association with surrounding peritoneum, and to the latter attach many of the axial muscle fibers heavily surrounding the slender ribs. These attached fibers appear to maintain a tension on the wall of the air bladder; cutting of the fibers creates a resonance within the bladder, and results in its partial collapse.

The maximum duration of angelfish grunts obtained during this investigation occurred west of Turtle Rocks on 10 July (Fig. 8), when an adult black angelfish examining the hydrophone and butting gently at its rubber case suddenly gave vent to prolonged, rather moan-like sounds, each of .2-sec. duration, and swam toward an approaching fish of the same species. The two fish faced each other for a few moments, after which both came quietly to the hydrophone and finally swam off together. The interpretation of a recognition signal in the prolonged grunts was rather difficult to avoid, for prior to production of the longer grunts, the first fish produced shorter, sharper sounds during its examination of the hydrophone. On another occasion, 12 July in the same area, sounds similar to the shorter grunts



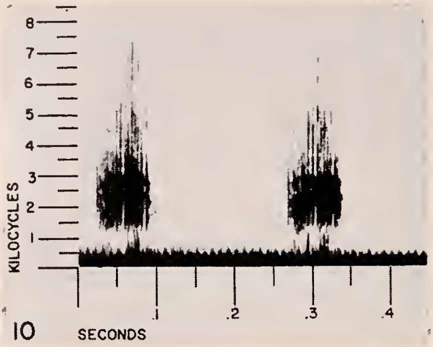
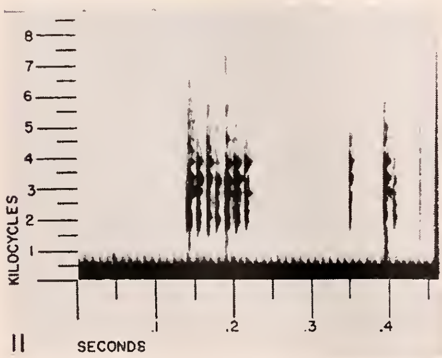


FIGURE 9. Toothplate stridulation of *Diodon hystrix* in aquarium.
FIGURE 10. Toothplate stridulation of *Spheroides spengleri* in aquarium.

were recorded as a pair of black angelfishes butted several times against a pair of cowfish, *Lactophrys tricornis* (L.), approximately 6 feet from the hydrophone.

No sounds were recorded from the single species of butterflyfish studied, *Chaetodon striatus* L., nor were sounds recorded from an immature specimen (4-inch, total length) of the French angelfish, *Pomacanthus paru* (Bloch).

Diodontidae, Tetradontidae (Fish, 1948, 1954; Burkenroad, 1931). The porcupinefish, *Diodon hystrix* L., and the puffer, *Spheroides spengleri* (Bloch), were so similar in acoustical behavior as to merit a single discussion (Table II). Both produce sound during and after inflation by stridulation of the toothplates, the sound being of a klaxon-like variety (Figs. 9, 10). Only feeding sounds were recorded from undisturbed individuals. Frequencies of greatest intensities are similar in chewing and stridulation noises, although these levels vary between the two species (Table II) and may be expected to vary with size. Only a single individual of each was recorded at Bimini. Observations on these species and on the common puffer, *Spheroides maculatus* (Bloch and Schneider) of the Woods Hole area suggest that the stridulatory sound is more readily elicited from smaller individuals than from full-grown animals.



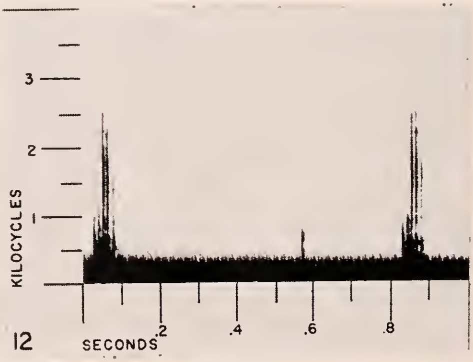
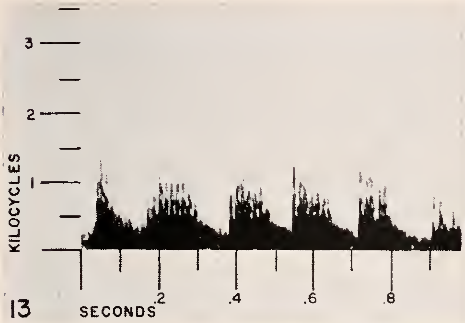
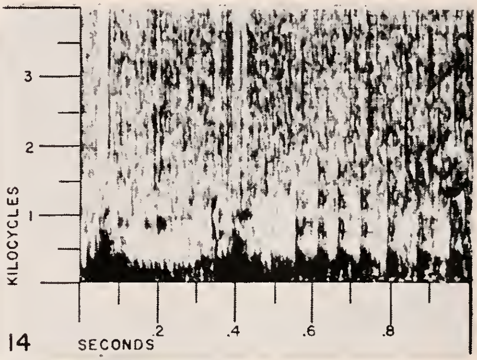


FIGURE 11. Pharyngeal tooth stridulation of *Haemulon sciurus* in aquarium.
FIGURE 12. Single thump-like sounds of *Holocentrus ascensionis* in cement tank.

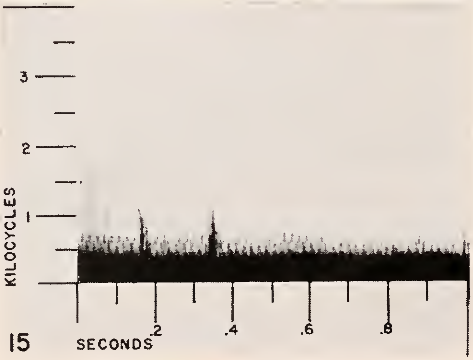
Haemulidae (Burkenroad, 1930, 1931; Fish, 1948, pp. 63–66; Schultz and Stern, 1948, p. 131). Although the fishes called grunts are notoriously noisy species of warmer marine waters, due to the rasping stridulation of the pharyngeal teeth, observations at Bimini indicated that not all species of the Haemulidae are equally important sound producers (Tables I and II). The haemulid rasp was not identified in any recordings made at sea; it was heard only from hand-held specimens of the blue-striped (Table II; Fig. 11) and yellow grunts, *Haemulon sciurus* (Shaw) and *H. flavolineatum* (Desmarest), respectively; no recordings of the latter were obtained. The observations of Burkenroad (1930) which related the sounds of *Haemulon* to movements of the pharyngeal teeth were confirmed, with the exception that the association between the dorsal pharyngeal teeth and the anterior end of the air bladder seems more important in resonating the rasping sound than does the varying relation between the air bladder and the lower pharyngeal teeth. The opercula are somewhat extended during sound production. Rubbing together of dissected pharyngeal toothplates produces a much fainter sound than that produced by the living fish.



13



14



15

FIGURE 13. Grunts of *Epinephalus striatus* in cement tank.

FIGURE 14. Two *E. striatus* grunts (left) and a volley of thump-like sounds of *H. ascensionis* west of Turtle Rocks. Snapping shrimp in background.

FIGURE 15. Snaps of male *Pomacentrus leucostictus* in aquarium.

Holocentridae (Fish, 1948). Sound production by the squirrelfish, *Holo-*

centrus ascensionis, said to have derived its name from its chattering call (D. de Sylva, personal communication), is characteristic of the western edge of the Great Bahama Bank (Table II; Fig. 12, 14). It was recorded only along, and mainly to the west of, the island and rock chain extending from North Bimini south to South Cat Cay (Tables III and IV; Fig. 16). In rocky areas of the bottom along a relatively narrow area probably extending not far below the limits of visibility from the surface, squirrelfish are characteristically found in the daytime each hovering near a depression approximately large enough to receive a single fish. Generally living in higher parts of ledges than the grouper, an occasional squirrelfish inhabits a rocky fissure with *E. striatus.* Collection of squirrelfish in traps up to a mile east of Turtle Rocks and occasional sightings in deeper reaches east of the Bimini-Cat Cay chain indicated that the species may move beyond its more common daytime habitat along the outer face of the Bank. Holocentrids are mainly nocturnal in their habits (Barbour, 1905, p.119; Randall, 1955, pp. 33, 38) and at night their local distribution may be considerably more dispersed than during the daytime, although the species considered here is said to return to the same hole each day (Ray and Ciampi, 1956, p. 207).

TABLE III

Grouper and squirrelfish sound production along the western edge of the Great Bahama Bank in the Bimini area

Recording station	Duration of recording	Calls/minute		
		Grouper	Squirrelfish	Both
1) At 15 fa. depth W of Moselle Shoal, drifting N	13 min.	.08	0	.08
2) At 25 fa. depth SW of (1), drifting N	18 min.	.4	0	.4
3) W of gap between Round and Turtle Rocks	6 min.	1.0	5.2	6.2
4) W of Turtle Rocks	5 min.	.2	7.2	7.4
5) W of Turtle Rocks	11.5 min.	.8	6.3	7.1
6) W of Turtle Rocks	45 min.	1.9	9.5	11.4
7) W of Turtle Rocks, drifting SE	14 min.	.6	3.4	4.0
8) W of Triangle Rocks, drifting NE	10 min.	1.3	3.3	4.6
9) West of Triangle Rocks	13 min.	1.7	3.2	4.9
10) W of gap between Piquet and Triangle Rocks	15 min.	2.5	8.6	11.1
11) W of Piquet Rocks	12 min.	2.4	4.1	6.5

Average number of calls/minute (11 stations):

Grouper	1.2
Squirrelfish	4.6
Both	5.8

Squirrelfish sounds are produced by contractions of body wall musculature against the rather firm-walled air bladder which is closely associated with the rib cage. The first three ribs are expanded, flattened and thinned dorsally, and are intimately associated with the air bladder wall; more posterior ribs are easily separated from the bladder. Physiological stimulation indicated that the musculature chiefly responsible for sound production was that attaching to the dorsal portions of the first three ribs, which appear to serve as drumheads. The structure of the air bladder, and its close relationship with the auditory region of the skull, have been described by Nelson (1955).

The thump-like sounds of squirrelfish may be repeated singly at irregular intervals or produced in rapid volleys; they are more sharply peaked on vibration frequency analysis than are the sounds of angelfish and grouper. When produced by startled or handled squirrelfish, the sounds are volleyed in bursts of 4 to 20. Singly-produced sounds are of longer duration than those produced in volleys; these singly-produced sounds are not dissimilar to the ear from the sounds of Nassau grouper, although they cover a greater frequency span than do those of the grouper when both are recorded at the hydrophone.

TABLE IV

Grouper and squirrelfish sound production over the Great Bahama Bank in the Bimini Area

Recording station	Duration of recording	Calls/minute		
		Grouper	Squirrelfish	Both
1) On Moselle Shoal	23 min.	.04	.06	.1
2) Over wreck ENE of North Rock	27 min.	.4	0	.4
3) At North Rock, E side	27.5 min.	.04	.2	.2
4) At Crossing Rocks, W side	37.5 min.	.1	1.0	1.1
5) In Bimini Harbor entrance, drift to N	6.5 min.	0	.6	.6
6) 50′ off S. Bimini, W side	13 min.	.7	.2	.9
7) At Round Rock, W side	8 min.	1.0	0	1.0
8) At Round Rock, E side	9 min.	.3	0	.3
9) On line between Turtle and Round Rocks, drift NW	10 min.	.9	1.9	2.8
10) Same station	30 min.	1.4	1.8	3.2
11) North end of Turtle Rocks, E side	4 min.	0	0	0
12) Same station	3 min.	0	0	0
13) On line between Triangle and Turtle Rocks, drift NE	12 min.	.4	2.2	2.6
14) Concrete shipwreck, E of Turtle Rocks	9 min.	0	0	0
15) Triangle Rocks, E side	11 min.	0	.6	.6
16) Dollar Harbor	5.5 min.	0	0	0
17) Wedge Rocks, NE side	8 min.	.8	1.4	2.2

Average number of calls/minute (17 stations):

Grouper	.3
Squirrelfish	.5
Both	.8

Undisturbed squirrelfish confined in laboratory aquaria and outdoor tanks remained silent during listening periods of up to three hours; confined specimens produced sounds only when handled, fed or startled. Quickened swimming during feeding was characterized by sound production of a considerably lower intensity than that produced during handling of the fish. Sudden startling of confined squirrelfish generally resulted in volleys of sound production.

At sea, squirrelfish were sometimes heard when no individuals were sighted, but the acoustical behavior of the species was characteristic when specimens were approached by the suspended hydrophone at drift stations. This behavior included

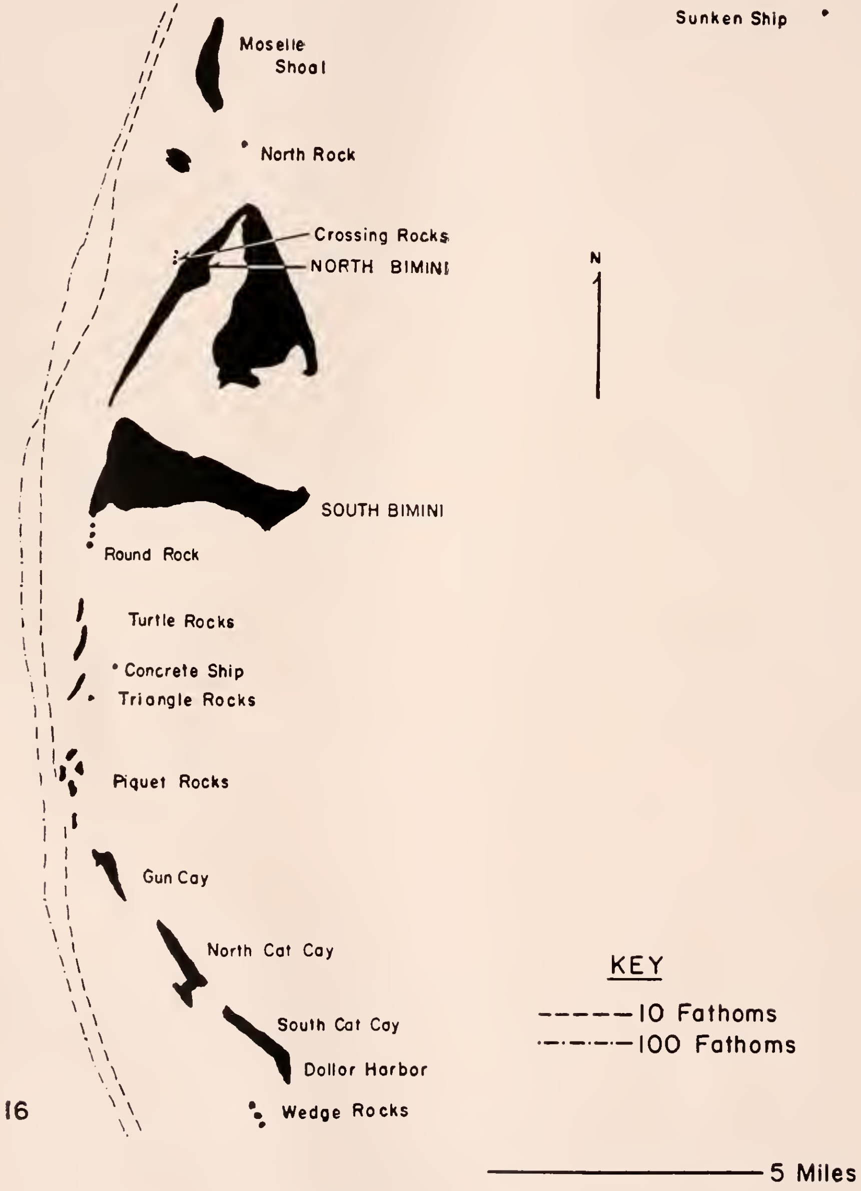

Sunken Ship
Moselle Shoal
North Rock
Crossing Rocks
NORTH BIMINI
N
SOUTH BIMINI
Round Rock
Turtle Rocks
Concrete Ship
Triangle Rocks
Piquet Rocks
Gun Cay
North Cat Cay
KEY
10 Fathoms
100 Fathoms
South Cat Cay
Dollor Harbor
16
Wedge Rocks
5 Miles

FIGURE 16. The Bimini area from Moselle Shoal to South Cat Cay.

erection of the spiny fins, adjustment of position so that an eye was directed toward the approaching hydrophone, movement toward a rocky depression near which the fish hovered, and production of the call in volleys of 3 to 20 individual pulses. The continuing approach of the hydrophone resulted in the fish's moving into its rocky shelter.

Pomacentridae (Fish, 1948, pp. 59–63). The sharp but rather faint knocks or snaps produced probably only by males of the demoiselle, *Pomacentrus leucostictus* (Mueller and Troschel), were recorded when a given individual suddenly dashed from cover to pursue other individuals approaching its place of concealment (Table II; Fig. 15). These were heard on several occasions. The behavior was similar to that of a sciaenid, *Corvina nigra* (Bloch) described by Dijkgraaf from Naples (1947).

Serranidae (Fish, 1954, pp. 36–44). The air bladder of the Nassau grouper, *Epinephalus striatus*, is thin-walled and lacks intrinsic muscles, yet this species equals the squirrelfish in its importance as a source of marine sound in the Bimini area (Table II; Figs. 13, 14). Contractions of body wall musculature appear to be responsible for the sound production, while the unusually heavy peritoneum stretched over the air bladder and adjacent organs apparently acts as the sounding board. Appropriate stimulation of opened fishes brought about some sound production, even after deflation of the air bladder. The strong contractions of body wall musculature accompanying production of the call are visible externally, and are easily elicited by startling or handling the fish in laboratory tanks. Sounds similar to those produced on alarm, but of a somewhat lower intensity, are produced during quickened swimming accompanying feeding.

Like the squirrelfish, the Nassau grouper inhabits the underwater ledges west of the Bimini-Cat Cay chain of rocks and island (Fig. 16). Its distribution is broader from east to west than that of the squirrelfish, since it was sighted where rocky fissures occurred in an otherwise sandy bottom in the Bimini lagoon, and it was seen and recorded in a shipwreck approximately 11 miles northeast of North Bimini Island on the Great Bahama Bank (Station 2, Table IV; Fig. 16). Unidentified sounds thought to originate from serranids were recorded at depths exceeding 15 fathoms west of North Bimini Island (Stations 1 and 2, Table III), from which depths serranids have been obtained at Bimini (Scholander *et al.*, 1951; Scholander and van Dam, 1954).

The Nassau grouper tends to be more secretive in its habits than the squirrelfish, ordinarily lying deeper in rocky fissures or well beneath overhanging ledges on the outer faces of which squirrelfish are more frequently seen. In acoustical behavior, the two are similar. As the suspended hydrophone approaches a grouper, the fins are erected and the fish retreats to cover, accompanying the retreat with the characteristic vibrant grunts. Like the call of the squirrelfish, that of the grouper was sometimes heard when no individual was sighted. The call is a characteristic marine sound of the area immediately west of the Bimini-Cat Cay chain.

The sound of the rock hind, *Epinephalus adsencionis* (Osbeck) (Table II), while of markedly lower intensity than that of the Nassau grouper, is produced under similar circumstances in laboratory tanks and is of similar characteristics. It was never specifically recognized during recording at sea. No sounds whatever were recorded from a captive *Promicrops itaiara* (Lichtenstein) weighing in the neighborhood of 300 pounds, although the fish on one occasion very nearly swallowed the hydrophone.

Distribution of Grouper and Squirrelfish Sounds in the Bimini Area

The distribution of squirrelfish and Nassau grouper in the Bimini area has been summarized above. Determination of this distribution was based on both visual and aural observations. The sounds of these species were selected to explore the possibility of determining the distribution of sonic species by aural means alone.

The total area of observation extended approximately 20 miles along the northwest edge of the Great Bahama Bank (Fig. 16), from Moselle Shoal on the north to Wedge Rocks on the south, and in an east-west direction from the location of a sunken freighter on the Great Bahama Bank (25° 49′ N, 79° 7′ W) 10.7 miles on a heading of 75 degrees from the northern tip of the Bimini Islands, to a station over an approximate depth of 150 feet southwest of Moselle Shoal. Recordings were made at 28 stations (Tables III and IV), all but two (Stations 1 and 2, Table III) being approximately on or within the 6-fathom line to permit observation of fishes during recording. Table III includes those stations, fixed and drift, at which recordings began on or were wholly confined to the edge of the Great Bahama Bank, to the west of the Bimini-Cat Cay chain; Table IV includes those stations located further in upon the Bank, in most cases immediately to the east of the Bimini-Cat Cay chain. The stations are in each case listed in order from north to south, and are referable to Chart No. 1854 of the United States Hydrographic Office, and to U. S. Coast and Geodetic Survey Chart No. 1112.

While not each call ascribed to either grouper or squirrelfish in Tables III and IV has been analyzed by vibration frequency analysis, a broad sample of recordings made at sea has been thus treated, and the results have indicated that the interpretations of recordings upon which Tables III and IV are based are correct. In erecting Tables III and IV, a rapid volley of either grouper or squirrelfish sounds has been characterized as a single call. Closely consecutive calls of the same species but of different individuals are usually interpretable as such on the basis of intensity differences, due to the origins' being at different distances from the hydrophone.

It will be noted in Table III that, although squirrelfish calls were lacking in the deepest stations recorded (1 and 2), along the sloping edge of the Bank they predominated over grouper calls by a factor of nearly 4. Moving of the listening station from the slope side of the Bimini-Cat Cay chain immediately to the Bank side (Table IV) resulted in an abrupt drop in incidence of calling by both species, but particularly by the squirrelfish. On the Bank itself, where squirrelfish were much less frequently sighted, their calls predominated over grouper calls by a factor of less than 2. The calling of these two species together was over 7 times as frequent along the edge of the Bank as to the east of its edge (Tables III and IV). The difference in incidence of calling between slope and Bank sides of the Bimini-Cat Cay chain is exemplified especially by comparison of Table III, Stations 4, 5 and 6, with Table IV, Stations 9, 10, 11 and 12. Short moves of the listening station resulted in marked differences in the incidence of underwater biological sounds.

Although not indicated by the data presented in Tables III and IV, observations at drift stations (Table III, Stations 7 and 8; Table IV, Stations 9 and 13) indicated that as the hydrophone moved over alternately sandy and rocky bottom, the incidence of calling rose markedly as the boat moved over underwater ledges and fell over the sandy stretches; the incidence of calling thus provided an indication as to the type of bottom beneath the boat, and correlated with sightings of grouper and squirrelfish.

OTHER UNDERWATER SOUNDS OF THE BIMINI AREA

Sounds most frequently heard during the underwater listening in the Bimini area, other than those described, are (1) the snapping and crackling characteristic of tropical seas, generally attributed to snapping shrimp, but actually indistinguishable by methods commonly employed from the sounds of some stomatopods (Johnson, *et al.,* 1947; Moulton, 1957); (2) a rattling sound like that produced by the spiny lobster, *Panulirus argus* (Moulton, 1957); and (3) another unidentified rattling sound with its predominant frequencies lying between .5 and 1.3 kc., each pulse being of .02 second duration, and repeated at intervals of approximately .12 second in volleys of varying length. In addition to these, a distinctive series of sounds was recorded twice the same day (12 July 1956 at Stations 6 and 9, Table IV). At Station 6, the sound was a buzz-like whine singly produced; at Station 9, the same sound was preceded by a number of sharp metallic raps and was followed by a number of brief chirps of somewhat lower frequency than the raps. The metallic raps were so similar to the sound of pounding on a steel hull that two observers, prone at the glass panels, concluded a vessel to be bearing down on the listening post. Since the listening boat was quiet except for water noise along the hull, and since no other boats were within view to the horizon, it is assumed that the sounds were of biological origin, but the source is unknown. They do not correspond with known cetacean sounds (Mr. William Schevill, personal communication).

DISCUSSION

The behavior of the Nassau grouper and squirrelfish in the Bimini area furnishes a marked exception to the generalization (Fish, 1954, p. 7) that fishes in the field are silenced by strange contacts. Both of these species were obviously stimulated to active sound production by approach of the boat and suspended hydrophone at drift stations over shallow waters. The spiny fin erection and movement toward concealment of these species upon approach of the hydrophone were strongly suggestive that the sounds described are related to self-protection, a probability further suggested by production of the same sounds during handling of these fishes. Although some fishes may use echo-location (Griffin, 1955), there is no evidence at present of its being involved in the cases under discussion. Circumstances surrounding production of grouper and squirrelfish calls, as observed at Bimini, were similar to those that surround production of sea robin grunts (Moulton, 1956b). While the grunts are readily produced during handling by most specimens of the sea robins common at Woods Hole, *Prionotus evolans* (L.) and *P. carolinus* (L.), the grunts are also produced by sea robins contained together in live cars and living on the bottom. Sea robin grunts are not, however, so easily stimulated by startling as are the sounds of Nassau grouper and squirrelfish which readily produced sounds recorded at sea after periods of confinement of two weeks in laboratory aquaria.

The squirrelfish is the most significant producer of underwater sound among fishes in the Bimini area, and if the 20-mile extent of the Bank area studied may be considered typical of the whole, of the edges of the Great Bahama Bank generally. In view of this significance, Fish's (1948, p. 44) estimate of the sonic importance of the Holocentridae as "probably none" must be rejected. Since holocentrids are of wide distribution in tropical and sub-tropical waters, it seems probable that their sonic importance extends to other waters than those of the Bimini area.

The acoustical behavior of the angelfishes (Chaetodontidae) has not been hitherto adequately described, but there can be little doubt that the behavior of the single specimen recorded at Turtle Rocks on 10 July furnished evidence of a call accompanying recognition behavior in this species. The black angelfish which is most common of the angelfishes in the Bimini area, has a tendency to examine underwater objects (hydrophone, swimming cowfishes), such examination being accompanied by sounds of briefer duration than those proposed as a part of recognition behavior. The black angelfish is usually observed in pairs during July and August at Bimini. It seems likely that chaetodontids may contribute to underwater sound in other tropical and sub-tropical areas, although they are not discussed among sound-producing fishes of the Pacific by Fish (1948).

Further evidence of the tendency of captive fishes to become silent unless disturbed was provided by all species studied during the summer of 1956. The only species to produce sound spontaneously in laboratory aquaria during listening periods of up to three hours, other than those produced during feeding and being startled, was the small pomacentrid, *Pomacentrus leucostictus*, probably a male, as it pursued other individuals encroaching on its hiding place.

As is the case along the northeastern coast of the United States where the most significant fish producers of underwater sound which have been identified (sciaenids, triglids, and batrachoidids) are fishes using, rather than skeletal stridulatory mechanisms, muscles in close association with the air bladder (Tower, 1908; Fish, 1954; Moulton, 1956b), a holocentrid, a serranid and a chaetodontid are the most frequently calling fishes of the area immediately about the Bimini-Cat Cay chain. Of the former, however, both sea robins and toadfish produce sounds with muscles intrinsic to the air bladder, while all three of the most important calling fishes of the Bimini area use muscles extrinsic to the air bladder in producing their sounds.

The structural specializations and behavioral patterns of the sound producing species studied at Bimini have provided further striking evidence of the significance of sound in the biology of the species concerned, and the consistent incorporation of sound production into behavioral patterns observed in the clear water about Bimini (Nassau grouper, squirrelfish, angelfish) strengthens a conclusion that sound is of significance to the species concerned. Yet, as has repeatedly been affirmed, clear evidence of effectiveness of the sounds concerned in modifying the movements of fishes in nature is still lacking, although Tavolga (1956) has observed the females of a goby to demonstrate increased activity during sound production by the male in breeding. The sounds of all calling species studied at Bimini are produced by both sexes, except for the snaps of *Pomacentrus leucostictus*.

Experiments of playing into the water artificial sounds and recordings of natural sounds such as those that have elsewhere modified fish behavior (Moulton, 1956a, 1956b; Tavolga, 1956) had no notable effect in the Bimini area. During all listening at sea in the Bimini area, all during daylight, sound production was prolific or rare, depending on the distribution of the species concerned and not on alternating periods of quiet and of sound production which seem to characterize production of the staccato call of sea robins at Woods Hole (Moulton, 1956b). Therefore, these experiments are not reported in detail. The Nassau grouper and squirrelfish were never observed in areas where their calls were not heard at Bimini.

The most characteristic component of background noise in the Bimini area is the "crackle" so characteristic of warmer seas, and which has generally been ascribed to snapping shrimp (Johnson *et al.*, 1947). By the analysis methods used, this noise in the Bimini area presents components cumulatively spanning the frequency range examined (up to 8 kc.). The invertebrates largely responsible are a common stomatopod (*Gonodactylus oerstedi*) and several kinds of snapping shrimps, including *Alpheus armatus* and *Synalpheus* spp. (Johnson *et al.*, 1947; Pearse, 1950; Moulton, 1957). The sound spectra obtained during this study from recordings of feeding and of stridulation sounds of various fishes indicate that in regions where producers of such sounds are numerous, they may contribute extensively to the spectrum of underwater sounds generally attributed to invertebrate sound producers.

I am much indebted to Dr. R. H. Backus for constructive criticism of the manuscript of this paper. I am also indebted to Mr. Donald de Sylva for assistance in identification of several of the fishes studied, and to Mr. E. R. Powell for assistance with the illustrations. Use of the generous facilities of the Lerner Marine Laboratory of the American Museum of Natural History is gratefully acknowledged.

SUMMARY

1. On the basis of observations and recordings at sea and in the laboratory, the acoustical behavior of 13 species of Bahamian fishes is described, and their sounds are defined. Twenty-six species producing no calls in the course of this study are specified.

2. The most important sound-producers among fishes of the Bimini area are the squirrelfish, *Holocentrus ascensionis,* and the Nassau grouper, *Epinephalus striatus*. Their characteristic sounds may be anticipated when these species encounter a strange object at sea, and probably generally during the daytime along the edges of the Great Bahama Bank.

3. A single observation has indicated that calling is a component of recognition behavior in the black angelfish, *Pomacanthus arcuatus*. The families Chaetodontidae and Holocentridae should be added to lists of fish families containing calling members.

4. The usefulness of underwater listening in studying the distribution of some calling fishes has been demonstrated in the cases of the squirrelfish and Nassau grouper.

LITERATURE CITED

Barbour, T., 1905. Notes on Bermudian fishes. *Bull. Mus. Comp. Zool.*, **46**: 109–134.

Bridge, T. W., 1910. Fishes. Cambridge Natural History, 7: 139–537. Macmillan and Co., Ltd., London.

Burkenroad, M. D., 1930. Sound production in the *Haemulidae*. Copeia, 1930, No. 1: 17–18.

Burkenroad, M. D., 1931. Notes on the sound producing marine fishes of Louisiana. Copeia, 1931, No. 1: 20–28.

Dijkgraaf, S., 1947. Ein Töne erzeugender Fisch im Neapler Aquarium. *Experientia,* **3**: 493.

Dobrin, M. B., 1947. Measurements of underwater noise produced by marine life. *Science,* **128** (**105** N.S.): 19–23.

Evermann, B. W., and M. C. Marsh, 1900. The fishes of Porto Rico. *Bull. U. S. Fish Comm. for 1900:* 1–350.

Fish, M. P., 1948. Sonic fishes of the Pacific. ONR Contr. N6 ori-195, T.O. 1, Tech. Report No. 2.

Fish, M. P., 1954. The character and significance of sound production among fishes of the western North Atlantic. *Bull. Bingham Oceanographic Collection,* **14**; Art. 3: 1–109.

Fish, M. P., A. S. Kelsey, Jr. and W. H. Mowbray, 1952. Studies on the production of underwater sound by North Atlantic coastal fishes. *J. Mar. Res.*, **11**: 180–193.

Griffin, D. R., 1955. Hearing and acoustic orientation in marine animals. Papers Mar. Biol. and Oceanogr., Deep-Sea Research, suppl. to Vol. 3, pp. 406–417.

Johnson, M. W., F. A. Everest and R. W. Young, 1947. The role of snapping shrimp (Crangon and Synalpheus) in the production of underwater noise in the sea. *Biol. Bull.*, **93**: 122–138.

Moulton, J. M., 1956a. The movements of menhaden and butterfish in a sound field. *Anat. Rec.*, **125**: 592.

Moulton, J. M., 1956b. Influencing the calling of sea robins (*Prionotus* spp.) with sound. *Biol. Bull.*, **111**: 393–398.

Moulton, J. M., 1957. Sound production in the spiny lobster *Panulirus argus* (Latreille). *Biol. Bull.*, **113**: 286–295.

Moulton, J. M., and R. H. Backus, 1955. Annotated references concerning the effects of man-made sounds on the movements of fishes. Fisheries Circ. No. 17, Dep't of Sea and Shore Fisheries, Augusta, Maine.

Nelson, E. M., 1955. The morphology of the swim bladder and auditory bulla in the *Holocentridae*. *Fieldiana: Zoology,* **37**: 121–130.

Pearse, A. S., 1950. Notes on the inhabitants of certain sponges at Bimini. *Ecology,* **31**: 149–151.

Randall, J. E., 1955. Fishes of the Gilbert Islands. Atoll Research Bull., No. 47, Pacific Science Board, Natl. Acad. of Sci., National Research Council, Washington.

Ray, C., and E. Ciampi, 1956. The Underwater Guide to Marine Life. A. S. Barnes and Co., New York.

Scholander, P. F., C. L. Claff, C. T. Teng and V. Walters, 1951. Nitrogen tension in the swimbladder of marine fishes in relation to the depth. *Biol. Bull.*, **101**: 178–193.

Scholander, P. F., and L. van Dam, 1954. Secretion of gases against high pressures in the swimbladder of deep sea fishes. I. Oxygen dissociation in blood. *Biol. Bull.*, **107**: 247–259.

Schultz, L. P., and E. M. Stern, 1948. The Ways of Fishes. D. Van Nostrand Co., Inc., New York.

Tavolga, W. N., 1956. Visual, chemical and sound stimuli as cues in the sex discriminatory behavior of the gobiid fish *Bathygobius soporator*. *Zoologica,* **41**, Part 2: 49–64.

Tower, R. W., 1908. The production of sound in the drum-fishes, the sea-robin and the toadfish. *Annals N. Y. Acad. Sci.*, **18**: 149–180.

EFFECT OF PLANT HORMONES ON ULVA[1]

L. PROVASOLI

Haskins Laboratories, 305 East 43rd Street, New York 17, N. Y.

Föyn (1934a) in his early attempts to grow *Ulva lactuca* found that *Ulva*, like *Cladophora suhriana* (Föyn, 1934b), grows poorly in sea water enriched with nitrates and phosphates (Schreiber, 1927) and that the addition of soil extract to Schreiber's medium is necessary to obtain normal growth and the entire life-cycle. This medium ("Erdschreiber") later became the standard medium for growing marine flagellates in bacterized cultures (Gross, 1937; Parke, 1949).

Kylin (1941) employed *Ulva lactuca* to analyze the biological activity of different samples of sea water: he found that sea water at 70 meters depth is inadequate to support normal growth and that addition of nitrates, phosphates and trace metals made it suitable for the germination of the zoospores of *Ulva* and elicited as rapid growth of the germlings to the stage of 15–20 cells as did the "Erdschreiber."

Levring (1946), employing the same technique and test organism, formulated a synthetic sea water which, similarly enriched, allowed normal development of the germlings of *Ulva*, thus confirming, with a chemically defined medium, Kylin's conclusion. Levring's medium was the starting point for the formulation of several synthetic marine media which do not precipitate and are suitable for the cultivation of a number of marine and brackish algal flagellates in bacteria-free culture (Provasoli, McLaughlin and Droop, 1957).

Since Föyn, Kylin and Levring worked with bacterized cultures, I wondered if *Ulva*, when bacteria-free, would grow in mineral media or if it would require organic factors. Many other algae which, like *Ulva*, were previously cultured in Erdschreiber + bacteria, require, besides nitrates and phosphates, growth factors and trace metals when cultured in synthetic media without bacteria (Provasoli and Pintner, 1953; Sweeney, 1954; Lewin, 1954; Droop, 1955a, 1955b, 1957; Provasoli, 1957).

In exploratory attempts to grow *Ulva* in bacteria-free culture, I failed to obtain a typical foliaceous thallus but in trying to obtain it, I found that *Ulva* germlings respond to plant hormones.

MATERIAL AND METHODS

Bacteria-free cultures of *Ulva* were obtained by placing pieces of thallus on the surface of agar media containing various concentrations of an antibiotic mixture (1 ml. of the concentrated antibiotic solution contains: K penicillin G 12,000 units; chloramphenicol 50 μg.; polymyxin B 50 μg.; neomycin 60 μg.).

I recognized from the beginning the necessity of employing thalli free from epiphytic organisms: the pieces of thallus were selected and inspected under the dissecting microscope and, as an additional precaution, were cleaned by brushing

---

[1] Aided in part by Contract NR 163-202 with the Office of Naval Research.

the two surfaces with a thick, soft water-color brush. Even so, the epiphytes could not always be eliminated and several bacteria-free cultures of *Ulva* were infected with small diatoms (mainly *Nitzschia*).

The concentrated antibiotic mixture was sterilized by filtration through a glass filter, and 0.1-, 0.2-, 0.3-ml. aliquots were dispensed on the bottom of sterile Petri dishes; 20 ml. of sterile 1.4% agar media, kept at 45° C., were added and thoroughly mixed with the antibiotic by twirling. The pieces of *Ulva* thallus (5-mm. squares) were left on this agar for 7 days, removed aseptically with a spatula, placed in depression slides containing sterile media, cut in several narrow strips with an iridectomy scalpel, and transferred to liquid media. The pieces treated with the lower concentrations of antibiotics (0.1–0.2 ml. in 20 ml. of agar media) were infected with either bacteria, a pink yeast, or diatoms. All the pieces treated with 0.3 ml. of antibiotic mixture (final concentration of antibiotics per ml. of agar medium: K penicillin 200 units, and 1 μg. each of chloramphenicol, neomycin, and polymyxin) were bacteria-free but about ⅓ were infected by diatoms.

Several liquid media were tried: the most successful were ASW III and ASW 8 (Table I); both are enriched sea water media similar to, but richer than, Erdschreiber. ASW III (richer in organics) was employed in the early experiments; later I employed ASW 8 which allows better growth.

The cultures are carried in screw-cap tubes (125 × 20 mm.) with 10 ml. of medium; to avoid chemical contamination we employ plastic caps without liners. At first, *Ulva* was grown in continuous light (200 foot-candles; fluorescent tubes) at 18–20° C., later in alternate light (16 hours) and darkness (8 hours).

The sample of gibberellins was kindly supplied by Dr. Nickell, of Chas. Pfizer & Co., and the one of kinetin bought from the California Foundation for Biochemical Research.

RESULTS

The purified strips of thallus, when transferred to liquid media, began to produce thin filamentous germlings from their surface and looked like pincushions. That many zoospores were also set free was evident from the many small germlings that covered the walls of the test tubes.

The germlings arising from the thallus were round, thin, solid, and never became more than 2–4 mm. long; after two months they bleached, leaving a few intensely green spots which dotted their surface at random. The germlings on the walls of the tubes in certain media behaved similarly and produced a number of rhizoids, many of them colorless; in other media, the germlings developed only rhizoids and looked like stellate colonies or like an elongated root system in miniature. Pieces of bleached stubby germlings, or the stellate rhizoidal colonies, when transferred to new media, produce new germlings from the islands of intensely green cells which are scattered among the bleached tissues; these green cells remain dormant and viable for a year (longest time tried) in the old medium.

The germlings of the second generation underwent a similar cycle, they grew a few millimeters and later bleached partially; no zoospores were produced by these germlings. Serial transfers are carried out by removing the old germlings aseptically from the culture tubes, cutting them in pieces and inoculating the pieces in different media.

Föyn obtained normal development of the thallus, production of zoospores and gametes of *Ulva,* in bacterized cultures grown in Erdschreiber. ASW III, an Erdschreiber enriched with vitamins, liver extract, and carbon sources, allowed only the formation of germlings; the typical thallus was never obtained in bacteria-free cultures in this medium. The beginning of a thallus (the formation of two short, thick "rabbit ears" or a fan-like curly, thick, small thallus) was obtained in two of the tubes which were infected with a pink yeast or a diatom. However, re-infection of the axenic cultures of *Ulva* germlings with clonal cultures of the yeast or diatom failed to produce a thallus. Disappointed by the failure to obtain normal thalli, I re-examined Föyn's cultural methods and noted that he had found (1955) that only the northern European variety of *Ulva* can be grown in continuous light, that the southern variety (which he proposed to call *Ulva thureti*) bleached if grown in continuous light and that a normal thallus was formed only with a daily period of 6 or more hours of darkness. From then on, all cultures had an 8-hour

TABLE I

Media for Ulva

	Föyn's Erdschreiber	ASW III	ASW 8
Sea water	100 ml.	100 ml.	80 ml.
H_2O			20 ml.
$NaNO_3$	10 mg.		30 mg.
KNO_3		20 mg.	
$Na_2HPO_4 \cdot 12\ H_2O$	2 mg.		
K_2HPO_4		2 mg.	
Na_2 glycerophosphate			3 mg.
Mn (as Cl)		0.04 mg.	
Fe (as Cl)		0.01 mg.	0.05 mg.
P II metals*			3 ml.
Vitamin mix No. 8**		0.1 ml.	
Vitamin mix S. 3***			0.5 ml.
B_{12}			0.01 μg.
Liver 1:20†		1. mg.	
Soil extract	5 ml.	4 ml.	
Na H glutamate		50 mg.	
Glycine		50 mg.	
Tris (hydroxymethyl) amino-methane††		100 mg.	100 mg.
pH	8.0	7.5	8.0

* One ml. of P II metal contains: ethylenediamine tetraacetic acid, 1 mg.; Fe (as Cl) 0.01 mg.; B (as H_3BO_3) 0.2 mg.; Mn (as Cl) 0.04 mg.; Zn (as Cl) 0.005 mg.; Co (as Cl) 0.001 mg.

** One ml. of Vitamin mix No. 8 contains: thiamine HCl, 0.2 mg.; nicotinic acid, 0.1 mg.; putrescine 2 HCl, 0.04 mg.; Ca pantothenate, 0.1 mg.; riboflavin, 5.0 μg.; pyridoxine 2 HCl, 0.04 mg.; pyridoxamine 2 HCl, 0.02 mg.; para-aminobenzoic acid, 0.01 mg.; biotin, 0.5 μg.; choline H_2 citrate, 0.5 mg.; inositol, 1.0 mg.; thymine, 0.8 mg.; orotic acid, 0.26 mg.; B_{12}, 0.05 μg.; folinic acid, 0.2 μg.; folic acid, 2.5 μg.

*** One ml. of Vitamin mix S. 3 contains: thiamine HCl, 0.05 mg.; nicotinic acid, 0.01 mg.; Ca pantothenate, 0.01 mg.; *p.*-aminobenzoic acid, 1.0 μg.; biotin, 0.1 μg.; inositol, 0.5 mg.; folic acid 0.2 μg.; thymine, 0.3 mg.

† Nutritional Biochemical Corporation, Cleveland, Ohio, U. S. A.

†† "Sigma 7–9 biochemical buffer," Sigma Chemical Co., St. Louis, Missouri, U. S. A.

1

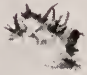
3

4

5

2

6


7

8

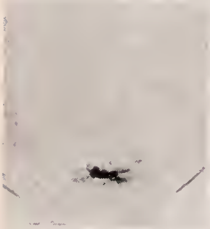
9

10

11

12

13


14

15

16

17

18

19

20

21

Figures 1–21.

dark period and 16 hours light but no thallus formed and the germlings bleached as before.

The repeated failure to obtain normal morphogenesis of the thallus in bacteria-free culture, in media similar to Erdschreiber, suggested that the failure was due to a lack of morphogenetic regulators; some of them, like indolacetic acid and gibberellin, can be produced by microorganisms. I tried various concentrations and combinations of adenine, indolacetic acid (IAA) and kinetin which influence growth and differentiation in higher plants.

In the first experiments we found that adenine by itself favored the production of more germlings from the dormant green cells and induced longer filaments; the best concentration was 3 mg.% (6 mg.% is inhibitory).

In the presence of 1 μg.% kinetin, indolacetic acid induced a large number of germlings whose length tended to increase proportionally with the concentration of IAA: 5 μg.% was the best. When 3 mg.% adenine was superadded, the most effective concentration of IAA was 10μg.%; 30μg.% inhibited the length and number of germlings.

In the presence of 5μg. IAA, increasing concentrations of kinetin also favor the elongation of germlings; the highest concentration tried (10 μg.%) induced the longest germlings obtained in these early experiments (Fig. 5). When adenine was superadded, the number of germlings induced by the combined action of IAA and kinetin is apparently not affected, but adenine completely inhibits the sharp elongation produced by 10 μg.% kinetin (Fig. 8). Perhaps in *Ulva* these morphogenetic determinants interact and have a limited specific action paralleling their activities on the tissues of higher plants.

At this point, we tried to substitute sea water media with synthetic mineral

---

EXPLANATION OF PLATE I

FIGURES 1–8. Medium ASW III: forty-five days' growth. The new growth is represented by the lateral filaments budding from the old pieces. FIGURE 1: ASW III alone. FIGURE 2: + kinetin 2.5 μg.%.

FIGURES 3–5. Kinetin curve: basal medium = ASW III + indolacetic acid 5 μg.%; FIGURE 3: + kinetin 1 μg.%; FIGURE 4: + kinetin 5 μg.%; FIGURE 5: + kinetin 10 μg.%.

FIGURES 6–8. Kinetin curve: basal medium = ASW III + indolacetic acid 5 μg.% + adenine 3 mg.%; FIGURE 6: + kinetin 1 μg.%; FIGURE 7: + kinetin 5 μg.%; FIGURE 8: + kinetin 10 μg.%.

FIGURE 9. ASW 8 medium + adenine 3 mg.% + kinetin 20 μg.%. Same as Figure 15 but after 120 days' growth. Note islands of green, resistant cells interspersed in the bleached tissue of the blade.

FIGURES 10–20. ASW 8 medium: sixty days' growth. FIGURES 10–13. Indolacetic acid curve: basal medium = ASW 8 + kinetin 10 μg.%; FIGURE 10: No addition; FIGURE 11: + indolacetic acid 5 μg.%; FIGURE 12: + indolacetic acid 10 μg.%; FIGURE 13: + indolactic acid 20 μg.%.

FIGURES 14–15. ASW 8 medium + adenine 3 mg.%; FIGURE 14: No addition; FIGURE 15: + kinetin 20 μg.%.

FIGURES 16–20. Gibberellins curve: basal medium = ASW 8 + indolacetic acid 5 μg.% + kinetin 10 μg.%. FIGURE 16: No addition; FIGURE 17: + gibberellins 1 μg.%; FIGURE 18: + gibberellins 10 μg.%; FIGURE 19: + gibberellins 40 μg.%; FIGURE 20: + gibberellins 100 μg.%: note that all the filaments are bleached and that only the rhizoids of the disc of attachment are still green.

FIGURE 21. Same as Figure 18, but after 120 days' growth; note the many knobby islands of resistant green cells interspersed on the bleached filaments.

Enlargement of all figures 2 × natural size.

media (Provasoli *et el.*, 1957) or with other types of enriched sea water to which we added the most effective hormone combination (*i.e.*, IAA 5 μg.% and kinetin 10 μg.%). ASW 8 was far better than both ASW III and the synthetic media, and was used from then on.

In ASW 8, formation of germlings and germling elongation was again favored by the combination of kinetin and IAA. With kinetin constant at 10 μg.%, only rhizoids were formed when IAA was absent; 10 μg.% IAA elicited longer germlings than 5 μg.% IAA after 30 days growth, but at this concentration the tips of the germlings became brown in 60 days and the germlings were completely brown in 90 days (Fig. 12). The combination of kinetin 10 μg.% and IAA 5 μg.% produced healthy green germlings which kept on growing and the tips began to flatten, as happens normally in nature, at an earlier stage (Fig. 11); at higher concentrations (20 μg.%) IAA inhibited and only rhizoids were produced (Fig. 13).

Gibberellin, superimposed on the favorable concentrations of kinetin and IAA, induced a dramatic elongation. As with IAA, concentrations of gibberellin approaching the lethal induce a more rapid elongation: thus gibberellin at 100 μg.% produced the longest and thinnest filaments at 30 days growth, but growth stopped at this time and the filaments were totally bleached at 60 days (Fig. 20); only the rhizoids of the attachment disc remained green. Gibberellin at 10 μg.% elicited maximum elongation; concentrations between 10 and 40 μg.% neither inhibited nor reduced the number of green islands of cells left when the germlings bleached at the end of growth (Fig. 21). Gibberellin at 1 μg.% seemed to produce a definite response as compared with the control, but this may be due to a difference in inoculum (compare Fig. 17 with Fig. 11).

So far, the response of *Ulva* to morphogenetic substances had been to induce few or many, and shorter or longer, atypical solid filaments—a far cry from what happens in nature; still, a beginning of blade formation could be detected in the flattening at the tips of the germlings grown in kinetin 10 μg.% + IAA 5 μg.%. An elongated flat blade, probably composed of two layers of cells and similar to the one normally occurring in nature, was obtained by the addition of 20 μg.% kinetin to 3 mg.% adenine (Fig. 15). Adenine alone and lower concentrations of kinetin (2.5, 5, 10 μg.%) + adenine were completely ineffective; only rhizoids and lumpy growth around the inoculum appeared; the atypical elongated germlings were completely lacking (Fig. 14).

Discussion

Though the studies of *Ulva in* bacteria-free culture are just beginning, two unexpected findings emerge: 1) a sea weed under our experimental conditions requires exogenous hormones for normal morphogenesis; 2) the thalli, typical and atypical alike, reach only an extremely small size as compared with the natural one, then bleach, but only partially, leaving islands of green cells which, when transferred to new medium, can originate new germlings.

Thuret (1878) described only two morphological types of cells in *Ulva:* the oblong cells constituting the major portion of the thallus and the rhizoids which make up the disc of attachment. The rhizoids are formed by "tubular cells" originating in the basal part of the foliaceous thallus: these cells elongate, push their tips downward between the two cell layers of thallus, reach the substratum to which the thallus is attached, and form a mat of filaments which anchors the thallus solidly.

Delf (1912) found that the tubular cells differ clearly from the other cells of *Ulva* in being multinucleate (they have 3–5 nuclei in the upper portion, few in the tubular portion and 2–5 nuclei in the rhizoidal portion). These observations were made on discs of *Ulva* growing on thalli of *Polysiphonia;* the material had been fixed in the early spring (*i.e.,* before the appearance of foliaceous thalli) yet the tubular cells were undoubtedly alive when fixed. Schiller (1907) believes that new germlings can originate from the rhizoids and Cotton (1910) and Delf (1912) postulate that the foliaceous part of the thallus of *Ulva* is annual while the disc of attachment is perennial.

Similarly, our experiments show that there are two types of cells: one which bleaches and dies easily, and a very resistant one. However, the resistant cells are located in two regions: 1) the disc of attachment, and 2) the erect elongated portion of the germling in which islands of cells remain green when the whole germling bleaches. Though both of these permanently green cells produce new germlings, they seem to have a different resistance to unfavorable conditions. At inhibitory concentrations of IAA (10 μg.%; Fig. 12) and gibberellin (100 μg.%; Fig. 20) no green islands appeared, all the cells of the erect part of the germling died, but the rhizoids remained green; at higher concentrations of IAA (20 μg.%; Fig. 13) the green islands of the inoculum did not produce new germlings but only a mat of rhizoids. It is most probable that the cells constituting the mat of the disc of attachment in our cultures are rhizoids, nonetheless we intend to test this hypothesis cytologically and see whether they are polynucleate. One would be tempted to consider that the cells of the green islands are also polynucleate because they are able to produce new germlings directly and without passing through the zoospore stage. However, they could also be morphologically identical with the oval cells which normally produce zoospores, but have a different potency. These cells do not appear only in the atypical germlings obtained in the laboratory; the original pieces of *Ulva,* from which our cultures derive, were small squares cut from the upper median part of the foliaceous thallus which is supposedly composed only of cells producing zoospores or gametes, yet not only zoospores were produced but a number of germlings originated directly from the piece of thallus which took the appearance, as noted, of a pincushion. Islands of permanently green cells in our cultures not only appeared in the atypical filamenteous germlings (Fig. 21), but also in the bleaching flat blade obtained with adenine + kinetin (Fig. 9). We can conclude then that another type of cell (different at least in its physiological potencies) exists among the oblong cells of the growing germlings and of the foliaceous thallus. These observations invite new studies on the morphology and potencies of the cells of *Ulva,* and on the localization and distribution in the various parts of the thallus of the various morphological and physiological types of cells. The resistant rhizoids of the attachment disc may prove the commonest and most valuable way of surviving winter and other hardships. The presence of other resistant cells in the foliaceous part of the thallus may be equally important ecologically in providing a more efficient way of spreading the species: pieces of thallus, fragmented by waves and transported by currents, can easily colonize distant sites far beyond the reach of the short-lived swimming zoospore stage.

Skoog and Miller (1957), in a penetrating review, conclude that regulation of growth may depend more upon the quantitative interactions than upon the qualitative action of the single plant hormones. This contrasts with the previous ideas that there are specific organ-forming substances and that "determination" is an irreversible loss in the regenerative abilities of cells and tissues.

We have not yet explored separately the action of each morphogenetic substance in its active range, nor the effects of kinetin at higher concentrations nor all the various combinations of morphogenetic agents. It seems, at this stage, that production of more germlings and, especially, the elongation into atypical germlings result from the combined action of indolacetic acid and kinetin; adenine and indolacetic acid appear antagonistic.

The narrow effective range of IAA is puzzling. Judging from the elongation of the atypical filaments, kinetin and gibberellin are not toxic over a wide range, while indolacetic acid is effective only in a narrow range (1 μg.% IAA is barely active, 5 are optimal, and 10 μg.% induce rapid growth followed by rapid death). The formation of a flat thallus, so far, has been obtained by combining adenine with kinetin, but in this combination kinetin is inactive up to 20 μg.%, while in combination with indolacetic acid it elicits elongation of atypical germlings at 10 μg.% (Fig. 5). So far, only adenine + kinetin have given normal growth while growth of atypical germlings results from the combined action of kinetin and IAA.

Distinguishing between specific actions, interactions, and mixed actions of plant hormones is a complicated task in higher plants: isolated specialized tissues—an artificial situation—may be misleading for morphogenetic conclusions; mixed tissues represent different potencies, while organs are too highly specialized and reflect the interdependency of many distinct tissues.

If other algae respond to plant hormones as one may expect, they may become excellent experimental material. The Chlorophyceae, because of their closeness to the primitive land plants, may be the best choice: they abound in species representing practically all the early steps of increasing structural complexity—the simple filament; different types of heterotrichous filaments; complex branched filamentous thalli in which the prostrate and the erect system may be unequally developed; thalli with specialized oogamy; and foliaceous thalli. With algae, one can work with *whole organisms,* and not with parts of highly evolved organisms artificially avulsed from the whole, simply by selecting species in order of increasing morphological complexity. The activity of plant hormones on *Ulva* raises the question of precisely where in the algal line of evolution toward the land plants, plant hormones were first employed as morphogenetic regulators.

Earlier studies on the action of indolacetic on unicellular algae seem unconclusive or negative. Preliminary experiments, done in collaboration with J. J. Pintner and K. Gold, show that several fresh water and marine unicellular algae and even the colonial *Volvox globator* do not respond to indolacetic acid, kinetin and gibberellin: growth rate, final growth and morphology are unchanged within the concentration range effective for *Ulva.* It is not surprising that flagellates which are considered morphologically the primitive form from which the vegetal and animal tendencies of the algae have evolved, do not respond to morphogenetic hormones. Hormones are concerned with the balanced growth of a cellular organism—how can one expect to find visible changes in an organism which has no cellular parts? The lack of effect on *Volvox* supports the generally held idea that this line is an evolutionary *cul-de-sac* and that *Volvox* is a colony of individuals. However, since the cell is the site of action of the hormones, "unicellular" algae may be the material of choice for studying the mode of action of plant hormones at the cellular level, but then, we need powerful specific antagonists to plant hormones.

It has been fortunate that plant hormones under our experimental conditions are indispensable for normal morphogenesis of *Ulva*. Quite likely this will hold for other media but, if it were not so, some nutritional factors upset the normal morphological development; the study of their role in morphogenesis could then allow a deeper insight into the action of plant hormones. ASW 8 allows better growth and the action of plant hormones is more evident in ASW 8 than in ASW III. The main difference between the two media is the presence in ASW III of an aqueous liver extract and soil extract, both of which introduce purines. Some purines, as adenine and kinetin, are important morphogenetic agents for *Ulva,* but it is conceivable that other purines may interfere with the normal processes of growth.

Definitive results can be obtained only by substituting for sea water a chemically defined medium to eliminate the unknown organic constituents of sea water.

The *Ulva* data suggest that these plant hormones may be as ecologically important for other sea weeds as vitamins are for phytoplanktonts. The auxins and gibberellins are microbial products (see Brian's review, 1957) and the unknown natural purines, which act like kinetin, may also be significantly contributed in natural waters by microbial action. It is possible therefore that the coastal zone, because of the land drainage which favors microbial growth, may never be so poor in plant hormones as to limit sea weed growth severely, but fluctuation in their level may control speed of growth and size of crop. This may be of economic importance to nations, like Japan and Ireland, which farm and use sea weeds extensively. To resolve these issues, not only are extensive pure culture studies needed but also convenient sensitive methods for assaying plant hormones in sea water.

SUMMARY

1. Bacteria-free *Ulva lactuca,* in sea water media enriched with vitamins, grows as atypical, short, filamentous germlings which do not develop into a foliaceous thallus. These filaments reach a few millimeters, then bleach, leaving a few islands of intensely green cells which, upon transfer to new media, produce new germlings.

2. The initiation of new germlings from these green islands and the length of the atypical filaments are increased by the combination of kinetin + indolacetic acid; adenine and indolacetic acid seem antagonistic. Conspicuous elongation of the filaments is promoted by the addition of gibberellins to the kinetin-indolacetic acid combination.

3. A normal flat blade was obtained so far only with adenine + kinetin.

4. The responses depend both on the interaction and concentrations of these morphogenetic agents: indolacetic acid is effective only in a very narrow range of concentrations and a blade is produced only with relatively high concentrations of kinetin.

5. The rhizoids of *Ulva* and the cells of the green islands can produce directly new germlings and are far more resistant to unfavorable conditions than the other

cells of the thallus which can originate zoospores or gametes. The morphogenetic and ecological significance of the resistant cells is discussed.

6. These responses of a relatively simply organized sea weed to plant hormones link even more tightly the green algae to the higher land plants.

7. The variety of evolutionary steps toward increased morphological complexity in the algae suggests that whole organisms, because of their relative morphological simplicity, may be valuable experimental material for studying the mode of action of plant hormones.

LITERATURE CITED

Brian, P. W., 1957. The effects of some microbial metabolic products on plant growth. *Symp. Soc. Exp. Biol.,* **11**: 166–182.

Cotton, A. D., 1910. On the growth of *Ulva latissima* in water polluted by sewage. *Kew Bull.*

Delf, F. M., 1912. The attaching discs of the Ulvaceae. *Ann. Bot.,* **26**: 403–408.

Droop, M. R., 1955a. Cobalamin requirement in Chrysophyceae. *Nature,* **174**: 520.

Droop, M. R., 1955b. A pelagic marine diatom requiring cobalamin. *J. Mar. Biol. Assoc.,* **34**: 229–231.

Droop, M. R., 1957. Auxotrophy and organic compounds in the nutrition of marine phytoplankton. *J. Gen. Microbiol.,* **16**: 286–293.

Föyn, B., 1934a. Lebenszyklus and Sexualität der Chlorophycee *Ulva lactuca* L. *Arch. f. Protistenk.,* **83**: 154–177.

Föyn, B., 1934b. Lebenszyklus, Cytologie und Sexualität der Chlorophycee *Cladophora suhriana. Arch. f. Protistenk.,* **83**: 1–56.

Föyn, B., 1955. Specific differences between northern and southern European populations of the green alga *Ulva lactuca* L. *Pubbl. Staz. Zool., Napoli,* **27**: 261–270.

Gross, F., 1937. Notes on the culture of some marine plankton organisms. *J. Mar. Biol. Assoc.,* **21**: 753–768.

Kylin, H., 1941. Biologische Analyse des Meerwasser. *Fysiogr. Sällsk. Förhandl.,* **11**: No. 21.

Levring, T., 1946. Some culture experiments with *Ulva* and artificial sea water. *Fysiogr. Sällsk. Förhandl.,* 16, No. 7:1–12.

Lewin, R. A., 1954. A marine *Stichococcus* sp. which requires vitamin B_{12}. *J. Gen. Microbiol.,* **10**: 93–96.

Parke, M., 1949. Studies on marine flagellates. *J. Mar. Biol. Assoc.,* **38**: 255–286.

Provasoli, L., 1957. Alcune considerazioni sui caratteri morfologici e fisiologici delle Alghe. *Boll. Zool. Agrar. e Bachich.,* **22**: 143–188.

Provasoli, L., J. J. A. McLaughlin and M. R. Droop, 1957. The development of artificial media for marine algae. *Arch. f. Mikrobiol.,* **25**: 392–428.

Provasoli, L., and I. J. Pintner, 1953. Ecological implications of *in vitro* nutritional requirements of algal flagellates. *Ann. N. Y. Acad. Sci.,* **56**: 839–851.

Schiller, J., 1907. Beiträge zur Kenntnis der Entwicklung der Gattung *Ulva. Sitzber. d. k. Akad. d. Wiss. in Wien.,* **116**: 1691–1706.

Schreiber, E., 1927. Die Reinkultur von marinen Phytoplankton und deren Bedeutung für die Erforschung der Produktionsfähigkeit der Meerwassers. *Wissench. Meeres unters. Helgoland N. F.,* **10**: 1–34.

Skoog, F., and C. O. Miller, 1957. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro. Symp. Soc. Exp. Biol.,* **11**: 118–131.

Sweeney, B. M., 1954. *Gymnodinium splendens,* a marine dinoflagellate requiring B_{12}. *Amer. J. Bot.,* **41**: 821–824.

Thuret, G., 1878. Études phycologiques. Analyses d'algues marines. Masson et Cie., Paris.

THE SO-CALLED "RECOVERY" PHENOMENON AND "PROTECTION" AGAINST X-IRRADIATION AT THE CELLULAR LEVEL

ROBERTS RUGH[1]

Marine Biological Laboratory, Woods Hole, Mass., and Radiological Research Laboratory, Columbia University, New York 32, N. Y.

There has been some evidence that if the sea urchin egg is allowed to remain unfertilized for several hours following x-irradiation, there will result a higher percentage of cleavage than when eggs are fertilized immediately after exposure. This has been designated as evidence of "recovery" of the egg by Henshaw (1932–1941). It presumes that such damage as is inflicted upon the egg can be rectified if the egg is given time.

Among higher forms many chemical agents have been examined to determine whether they might either "protect" the organism against x-irradiation lethality, or enhance the "recovery" from radiation insult. Among the first of the effective agents was cysteine (Patt, 1955) but more recently another —SH compound, namely cysteinamine,[2] has proven to be at least as effective. No drug has given complete protection but many have increased the dose necessary to kill a given percentage of the exposed animals, or to increase the probability of survival.

The mechanism of either the so-called "recovery" or of the chemical "protection" against x-irradiation has not been revealed. Since, among higher forms, the hematopoietic and reticuloendothelial systems are the more radiosensitive, and since the "protected" animals show quick regeneration of these systems, it has been presumed by some investigators that the so-called protective drugs somehow substituted for or masked the enzymes critically important in respiration. Since the sea urchin (Arbacia) egg showed the "recovery" phenomenon but did not have any hematopoietic system, it seemed appropriate to study the possible "protective" value of cysteinamine followed by x-irradiation and prior to fertilization of the Arbacia egg, and its possible effect on the "recovery" phenomenon.

MATERIALS AND METHOD

The eggs of Arbacia are readily available during the early summer and are easily fertilized to give nearly 100% cleavage and normal development. In every instance eggs from the same female were used for controls and for x-irradiation with and without benefit of the drug, so that there was no variation in the biological material. Fertilization or subsequent fixation of all the eggs of any single experiment was accomplished in 60 seconds, and in the same order in the various con-

---

[1] This work was done under Contract AT-30-1-GEN-70 for the Atomic Energy Commission.

[2] Cysteinamine is also known as cysteamine or beta-mercaptoethylamine or, commercially, as Becaptan hydrochloride. It was made available through Dr. W. Christiansen, representing the Labaz Laboratory, in Belgium.

tainers. The fixation was at stated intervals after insemination for the purpose of determining the cleavage percentage, and since the order of fixation was the same as the order of fertilization, and in the same elapsed time, the interval for all eggs between fertilization and fixation was the same.

It is obvious that when one deals with the cleavage percentage at a specified time after fertilization of an egg so temperature-sensitive as is the egg of Arbacia, that each set of data from the eggs of a single female on any particular day must be considered separately from other data similarly collected on other days for the simple reason of temperature variability, even though the range of temperature variation was very low. By maintaining controls for each set of data separately, effects of temperature fluctuation were obviated. This was considered to be more accurate than using a temperature control bath and averaging the data.

The eggs from a single adult Arbacia were removed by forceps and placed over cheesecloth in a beaker of filtered sea water. Within 15–20 minutes all of the mature eggs were dehisced from the ovaries and settled through the cloth into the beaker. When most of the eggs had settled on the bottom, the supernatant sea water was decanted off, and the eggs were transferred to a 500-cc. graduate which was filled with fresh, filtered sea water so that the eggs were thoroughly washed. This was repeated twice more. Finally, the eggs were diluted to 800 cc. of sea water and divided into two lots, one of which was considered the control lot. To the other was added 1 cc. of cysteamine from a freshly opened vial. In this way the concentration of eggs was the same in the control and experimental batches. The cysteamine was added within one-half hour of the beginning of irradiation so as to allow complete penetration into the egg. One cc. of cysteamine in 400 cc. of suspended eggs made a 0.0025 gram per cent solution which was non-toxic and in which eggs could be fertilized and would develop to plutei. With the above conditions, the variables were reduced to two, namely the level of exposure with or without the chemical agent.

Eggs in uniform concentration were placed in covered plastic fly boxes measuring 67 mm. in diameter and 20 mm. in depth. The controls were placed in one box and those in the chemical agent in the other, and the two boxes were superimposed during x-irradiation. When half the desired dose had been delivered, the position of the two boxes was reversed so that the slight difference in geometry of the two, with respect to the radiation, would be balanced out, and the exposure would be equated.

The x-irradiation facilities used were those provided by the Marine Biological Laboratory at Woods Hole, Mass., and consisted of two alternate-parallel x-ray tubes run at 182 KVP and 25 MA and having an equivalent filtration of 0.2 mm. of copper.[3] The output of the combined tubes at position "A" was 5,184 r/min. and at position "B" was 2,160 r/min. in air. In position "A" the distance between the targets was 18 cm. and at position "B" it was 29 cm. The maximum interval of exposure was 57.52 minutes but during this time there were brief interruptions during which eggs were removed. At all times a fan blew cool air over the dishes to dissipate any heat from the x-ray tubes. Should there have been any heat effect it would be the same for experimentals and controls. The exposures ranged from 25,000 r to 125,000 r.

---

[3] The author expresses here his appreciation of the efficient irradiation service rendered by Mr. Alan Brockway.

Fertilization of all eggs from a single experiment was accomplished within a period of less than one minute. At designated intervals following fertilization, samples of eggs were removed from the dishes by pipette and fixed in 10% formalin in sea water in Syracuse dishes to which had been added a small amount of acetic acid. Fixation was immediate. The order of fertilization was followed when the samples of eggs were fixed so that the time variable during the fertilization procedure was cancelled out by the same time interval and order of fixation. The percentage of cleavage was determined subsequently by counting groups of 200 eggs in each Syracuse dish, and the percentage was recorded to the nearest 5%. It was found that under the temperature conditions of the laboratory, fixation at six hours post-fertilization would include all the eggs which would ever enter cleavage, taking into consideration the matter of delay in cleavage which could be attributed to x-irradiation alone.

TABLE I

*"Recovery" in cleavage rate of Arbacia eggs following x-irradiation, due to delay in the time of fertilization**

Time: Fert. to fixation............	1⅓ hrs.				2⅓ hrs.				24 hrs. Blastula
Time: Irrad. to fertilization (hrs.)...	5	3	2	1	5	3	2	1	5
Controls	90	99	95	95	95	99	99	99	100**
25,000 r	75	85	60	10	85	99	90	90	100**
50,000 r	5	10	1	2	90	80	80	80	50
75,000 r	0	0	0	0	40	30	45	30	10
100,000 r	2	1	0	0	80	80	35	10	5

* Values in percentage cleaved eggs.

** See footnote No. 4.

Note: Maximum cleavage was achieved in eggs allowed to stand in fresh sea water for three hours after irradiation and before fertilization. After 100,000 r some 80% of the eggs did cleave in the time interval of two and one-third hours. The fact that at 75,000 r there was lower percentage of cleavage may indicate that some egg nuclei were functioning while at 100,000 r there may have been complete parthenogenesis.

EXPERIMENTAL DATA

The experimental data consist of percentage cleavage and development at stated intervals following delayed fertilization and also following irradiation in cysteinamine, as well as a combination of the two variables.

"Recovery": At average laboratory temperatures 50 per cent of the eggs of Arbacia will achieve the first cleavage within one hour after fertilization. If one examines fertilized eggs at one and one-third hours, the maximum percentage of cleavage will be observed. When eggs are x-irradiated and then fertilized by normal sperm, a delay in cleavage is observed, rather directly related to the level of irradiation. In the experiments reported here, eggs were x-irradiated from one

---

[4] The fact that there was a higher percentage of blastula than of cleavage is explained on the basis of delay in cleavage by the specific times of fixation for determining cleavage percentage. If time is not limited, the blastula percentages can be regarded as evidence of delayed but ultimate development. This apparent disparity occurred even slightly for the controls.

to five hours before insemination and the percentage cleavage was determined at one and one-third, two and one-third, three and twenty-four hours thereafter. The data are found in Table I below.

The term "recovery" has been used to describe the above phenomenon, since at any given time there is a higher percentage of cleavage in those eggs which had the greatest delay between x-irradiation and fertilization. The fact that even after 100,000 r the eggs do eventually cleave is of interest, indicating that the changes brought about by x-irradiation may be cytoplasmic, indirectly affecting the mechanisms of mitosis. In other words, the cytoplasm may show some evidence of "recovery." The mechanism for mitosis (*e.g.,* the chromosomes and spindle) could be contributed by the unirradiated sperm. Since none of the eggs x-irradiated above 50,000 r developed into plutei, it must be assumed that the damage to the egg nucleus is beyond "recovery," preventing normal development.

TABLE II

Cysteinamine "protection" of the Arbacia egg against x-irradiation. Effect on the time of the first cleavage

Date	Percentage cleavage (400 eggs) at 2.5 hrs. post-insemination							
	Controls		51,840 r		69,120 r		86,400 r	
	Sea wat.	Chem.	S.W.	Chem.	S.W.	Chem.	S.W.	Chem.
6/20	91	82		90	4	94	1	63
6/21	88	82	0	8	0	12	0	2
6/23	90	82	39	87	4	85	0	54
6/25	83	80	33	69	24	54	4	44
6/26			71	83	20	81	4	84
6/27			33	83	6	84	4	61
6/27			9	78	3	73	1	32
Averages:	88	82	31	70	9	69	2	48

Note: Any single experiment is complete in itself, but the data may vary with those from another day for reasons of temperature fluctuations alone. Thus, data on any horizontal line should be compared and it will be seen that in *every instance* there is a higher percentage of cleavage at 2.5 hours in eggs x-irradiated in the chemical than in the sea water alone. This is positive proof of qualified "protection" at the cellular level. The averages, below the table, have only relative significance but confirm the above.

Since there is no "recovery" of the developmental potentialities it may be possible to explain the phenomenon in terms of temporary interference with insemination and fusion of the protonuclei, or with the mechanics of mitosis, the degree of interference being related to the level of x-irradiation. It is not likely that this delay is due to any effects of the x-irradiated egg, exudates, or sea water *on the spermatozoa,* for their time span of activity in dilute suspensions is very short at best. It is more probable that the egg membrane, the cytoplasm, or even the elements involved in the kinetics of pronuclear fusion and mitosis may be temporarily altered so as to delay the progression of the sperm nucleus toward the egg nucleus. Whatever this effect of x-irradiation, it is reversible in time and even after 100,000 r some 80% of the eggs will achieve the first cleavage at least. To call this "recovery" is misleading, simply because the word generally implies much more than is demonstrated here. No egg exposed to more than 50,000 r x-rays ever "recovers" in the sense that it can develop past the critical stage of gastrulation. There is restoration of the conditions necessary for early mitosis only.

Chemical protection: Likewise the word "protection" must be qualified for in no permanent sense does the chemical "protect" animals (or eggs) against the effects of ionizing radiations, although it may lessen the effects. Nevertheless, if one x-irradiates Arbacia eggs in a tolerable solution of cysteinamine, then transfers them to filtered sea water and inseminates them, there is evidence that a higher percentage of eggs will cleave than in the "unprotected" controls, no matter what level of irradiation. Table II below illustrates this point.

TABLE III

The effect of time between irradiation and fertilization as well as immersion of eggs in cysteinamine during exposure, on the percentage of cleavage (200 eggs to nearest 5%)

Time: Irradiation to fertilization	Eggs fixed 2 hours after insemination							
	6 hrs.		5 hrs.		2 hrs.		1 hr.	
	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.
Control	100	100	100	100	100	100	100	100
50,000 r	60	90	40	90	25	100	1	90
75,000 r	5	95	2	95	0	90	0	30
100,000 r	10	80	0	90	0	2	0	0
125,000 r	0	30	0	30	0	0	0	0

Note: Experimental eggs were those immersed and irradiated in the chemical while the controls were x-irradiated in filtered sea water. In *every instance* where data are available, eggs x-irradiated while in the chemical show much better percentage of cleavage than did the controls. That both "protection" by the chemical and time for "recovery" were working is indicated by the fact that at 75,000 r we have a range of cleavage 30% to 95%, depending upon the time interval between irradiation and fertilization.

Cleavage percentage values include the 2- to 8-cell stages. At 24 hours those which had been chemically "protected" during x-irradiation were normal-appearing blastulae while the control eggs were disintegrated or abnormal blastulae. Even after 125,000 r 10 per cent of the experimentals were normal-appearing blastulae while the controls were 100 per cent abnormal.

When one combines the two variables of time between x-irradiation and fertilization as well as the presence of the so-called "protective" agent during x-irradiation, data of Table III are obtained.

When one delays examining the eggs until about three hours after insemination, further facts become clear. An exposure of 50,000 r does not prevent cleavage in the slightest, although there is a delay. Above this level of exposure there is a drop in ultimate cleavage percentage so that at 125,000 r the maximum level of cleavage is 50% at three hours after insemination in those eggs which had an interval of five hours between irradiation and insemination. A further delay in insemination (to six hours) was deleterious so that no eggs developed.

In every instance where eggs were x-irradiated in cysteinamine, there was higher percentage of cleavage than in the "unprotected" controls. In fact, the level of cleavage was the same as that of the controls or 100% even after 125,000 r, providing there was a delay in fertilization. *Thus, we see two forces acting in favor of the egg, with additive effects up to the point of five hours delay in fertilization.*

Those eggs which were inseminated within one hour of exposure to 125,000 r, while suspended in cysteinamine, showed 90% cleavage at three hours. This is certainly evidence of better "recovery" by means of the "protective" action of the drug than by time lapse between x-irradiation and fertilization. True, this was increased slightly to 100% by adding the time factor, but these figures are too close to be significantly different.

Table IV

The effect of time between x-irradiation and fertilization, as well as presence of cysteinamine during exposure, on the percentage of cleavage of Arbacia eggs

Time: Irradiation to fertilization	Eggs fixed 2 hours after insemination							
	6 hrs.		5 hrs.		2 hrs.		1 hr.	
	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.
Control	100	100	100	100	100	100	100	100
50,000 r	100	100	100	100	100	100	100	100
75,000 r	80	100	80	100	90	100	80	100
100,000 r	30	100	30	100	20	90	30	90
125,000 r	0	80	50	100	40	80	10	90

Note: There is evidence from these data that cysteinamine did not alter the tendency of eggs to "recover" if time was allowed between irradiation and insemination but that the two factors were additive.

At 24 hours all controls above 50,000 r were dead while those x-irradiated to this level in cysteinamine showed 100% ciliated motile blastulae (even after 125,000 r). After 48 hours a few from 50,000 r and 75,000 r plus cysteinamine developed into crude plutei. This would never happen with "unprotected" control eggs.

Immersion of eggs in cysteinamine following x-irradiation was invariably deleterious, the eggs going to pieces during the early attempts to cleave.

Discussion

The words "recovery" and "protection" when used in radiobiology must be qualified or clearly defined.

"Recovery" has been used to imply a return to the pre-irradiated state. It is defined as a "restoration to the normal state." It is very doubtful that this ever occurs following x-irradiation-induced morphological change at the cellular level. In a complex and multi-cellular mammal, for instance, there may be "recovery" in the sense that hair returns after epilation, lymphocytes appear after lymphopenia, and even the sterile testis may again become functional. Nevertheless, in every one of these examples it is more likely that there has been regeneration from un-damaged cells and that the originally damaged cells have been removed. The organism as a whole does have remarkable powers of restoration, but there is no evidence that the once damaged cells can themselves be "restored to the normal state."

There is no doubt (as Henshaw first pointed out) that a delay in fertilization after x-irradiation of the Arbacia egg will allow a greater percentage to undergo the early cleavages. This was confirmed up to a delay of three hours. However, ultimate cleavage was not improved and development was never achieved beyond the gastrula stage in eggs exposed to 50,000 r or more. Thus, the so-called "recovery" relates to counteracting somehow the delaying effects of x-irradiation on the mechanism of cleavage in the egg. It does not mean that the radiation-insulted egg can develop as does an unirradiated egg, a true recovery situation.

The word "protection" is also used variously (Bacq and Alexander, 1955). With mice, rats, guinea pigs, etc., it generally refers to the ability of a larger percentage of animals to survive a given dose of radiation, or to tolerate a larger dose than usual. The data are generally based upon a thirty-day survival. Certainly such animals exhibit most of the expected sequelae of x-irradiation such as shortening of life, higher incidence of cataracts, sterility, etc. It has been erroneously presumed that animals surviving thirty days after exposure are "normal" and the word "protection" is used.

Hollaender and Doudney (1955) found that when *E. coli* were x-irradiated in cysteinamine, they could tolerate twelve times as much of an exposure. That is, a dose of 60,000 r with the chemical had the same effect as 5000 r without its presence. Patt (1955) has reviewed the thesis that it is the sulfhydryl group (—SH) that is protective, by virtue of its ability to either protect or substitute for an enzyme, or to reactivate the enzyme after x-irradiation. The difficulty lies in the fact that there are many compounds containing the —SH group which have no protective value. Hollaender and Stapleton (1953) and Gray (1956) suggested that chemicals such as cysteine, entering the cell, require oxygen in order to be metabolized and thereby render the intracellular substance anoxic. But anoxia, while favoring survival of x-irradiated cells, probably does not alone explain the mechanism of cysteinamine protection.

It might be suggested here that the delay in initiation of cleavage is due to cytoplasmic effects of ionizing radiations on the egg, and those cytoplasmic effects are protected by such a chemical agent as cysteinamine. Certainly the results herein reported indicate that cleavage time is better "protected" by this chemical than by the time-lapse between x-irradiation and insemination. It is probable that the so-called "recovery" of the egg in time, with respect to initial cleavage and without benefit of chemical agent, is achieved largely by effects in the cytoplasm. One would tend to agree with Blum *et al.* (1951) that in addition to the cytoplasmic damage there is more serious damage, concerned with embryonic development, affecting the nucleoproteins. However, this is not manifest until the critical phase of gastrulation. It is of interest that the only eggs exposed to x-irradiation above 50,000 r which reached the recognizable pluteus stage were those which had been "protected" by cysteinamine and none of those benefited by a delay in insemination. This again indicates that the two mechanisms may act in a different manner but are not in conflict. In fact, they may be additive at lower levels of exposure.

Finally, it has been shown (see Bacq and Alexander, 1955) that glutathione plays a metabolic role in the process of cell division by effecting a fermentation within the cell which stimulates cell division. Specifically, cell division is not possible without the prior denaturation of protein by means of the —SH radical and the consequent reduction of the store of oxidized glutathione. Glutathione changes the oxidation-reduction level which in turn alters the fermentative metabolism that leads to cell division. The presence of thiol poisons in the cell, which could inhibit cell division, can be reversed by some of the —SH compounds, such as cysteine (Hammett, 1929) and presumably also by cysteinamine. That the —SH radical is vitally concerned in cell division has been well demonstrated.

There is no doubt that there is an increase in the soluble thiol compounds prior to cell division and also a high concentration of —SH radicals before, during and after mitosis. It is therefore conceivable that cysteinamine, like glutathione (Stern, 1956), might play a critical metabolic role in affecting mitosis. If the x-irradiated cell is given some time to repair the interference with the mitotic mechanism, or if the cell is provided with an excess of the —SH radical in the form of cysteinamine, the expected effect of cleavage delay caused by x-irradiation is not experienced. But, making available the —SH radical certainly does not allow true and full "recovery" nor is it fundamental "protection" of the cell since we would be ignoring herein all effects of x-irradiation on the chromosomes and genes which have to do with normal developmental processes. Therefore so far as development is concerned, there is neither "recovery" nor "protection."

Summary and Conclusions

1. The often used terms "recovery" and "protection" in radiobiology are specifically defined. As circumscribed, recovery at the cellular level does not occur following x-irradiation damage. Protection simply refers to better survival and in no way implies saving the exposed cell from the sequelae of x-irradiation insult.

2. The prior finding (of Henshaw) that a delay in insemination of Arbacia eggs following x-irradiation will allow for better initial cleavage percentage has been confirmed. However, it has been shown that there was no actual increase in ultimate cleavage percentage but rather, the x-irradiation factors which caused a delay in cleavage were neutralized. X-irradiated eggs never "recovered" because they could not develop through gastrulation to plutei.

3. Cysteinamine, if available to the Arbacia egg during x-irradiation, will counteract the delaying effect of x-irradiation on cleavage following insemination and will, in addition, allow further development. Some embryos will achieve the pluteus stage. This suggests that in addition to the apparent "recovery" by delay in insemination, the —SH cysteinamine must in some way reduce or prevent irradiation damage to the nucleus in some eggs, allowing them to become ciliated blastulae and even stunted plutei. They do not develop further than abnormal plutei following x-irradiations above 50,000 r, even with the benefit of the cysteinamine, so that protection of the egg to allow normal development did not occur.

4. Cysteinamine imposed on the Arbacia egg after x-irradiation was so deleterious that the early cleavage stages disintegrated rapidly even in concentrations which were tolerated well by unirradiated embryos. This may be due to the anoxia caused by cysteinamine.

5. It is concluded that "recovery" from x-irradiation damage defined as "restoration of the normal state" does not occur even with a delay in insemination. There may be some evidence of chemical nuclear "protection" to the extent that some eggs can develop to the early pluteus stage, but no further. This is hardly "protection" in the common usage of the word. It is concluded, therefore, that nuclear damage by x-irradiation is *irrevocable* and *irreparable and* that *neither "recovery" nor "protection,"* properly defined, *occurs at the cellular level following x-irradiation insult.*

LITERATURE CITED

Bacq, Z. M., and P. Alexander, 1955. Fundamentals of Radiobiology. Academic Press, N. Y.

Blum, H. F., J. C. Robinson and G. M. Loos, 1951. The loci of action of ultraviolet and x-radiation and photorecovery in the egg and sperm of the sea urchin *Arbacia punctulata. J. Gen. Physiol.,* **35**: 323.

Gray, L. H., 1956. A method of oxygen assay applied to a study of the removal of dissolved oxygen by cysteine and cysteamine. *In:* Progress in Radiobiology, ed. Mitchell, Holmes, Smith, Oliver and Boyd, London.

Hammett, F. S.,1929. The chemical stimulus essential for growth by increase in cell number. *Protoplasma,* **7**: 297–322.

Henshaw, P. S., 1932. Studies of the effect of roentgen rays on the time of the first cleavage on some marine invertebrate eggs. I. Recovery from roentgen ray effects in Arbacia eggs. *Amer. J. Roentgen. Rad. Therapy,* **27**: 890–898.

Henshaw, P. S., 1933. The effect of roentgen rays on the time of the first cleavage in marine invertebrate eggs. II. Differential recovery and its influence when different methods of exposure are used. *Radiology,* **21**: 533–541.

Henshaw, P. S., and D. S. Francis, 1936. The effect of x-rays on cleavage of Arbacia eggs: Evidence of nuclear control of division rate. *Biol. Bull.,* **70**: 28–35.

Henshaw, P. S., 1940. Further studies on the action of roentgen rays on the gametes of Arbacia punctulata (6 parts). *Amer. J. Roentgen. Rad. Therapy,* **43**: 899–933.

Henshaw, P. S., 1941. The induction of multipolar cell division with x-rays and its possible significance. *Radiology,* **36**: 717–724.

Hollaender, A., and C. O. Doudney, 1954. Studies on the mechanism of radiation protection and recovery in the cysteamine and β-Mercaptoethanol. *Symp. Radiobiol. Liege,* pp. 112–121, Butterworths, London.

Hollaender, A., and G. E. Stapleton, 1953. Fundamental aspects of radiation protection from a microbiological point of view. *Physiol. Rev.,* **33**: 77.

Patt, H. M., 1955. Remarks concerning sulfhydryl-protection against mammalian radiation injury. *Symp. Radiobiol. Liege,* pp. 105–109, Butterworths, London.

Stern, H., 1956. Sulfhydryl groups and cell division. *Science,* **124**: 1292–1293.

DEVELOPMENT OF PIGMENT IN THE LARVA OF THE SEA URCHIN, LYTECHINUS VARIEGATUS[1, 2]

RICHARD S. YOUNG[3]

Department of Zoology, Florida State University, Tallahassee, Florida

Very little is known concerning the origin of invertebrate pigment cells. Most invertebrate eggs contain varying amounts of carotenoid pigments, which are sufficient to mask the appearance of any new pigments. The unsegmented egg of the sea urchin *Lytechinus variegatus,* however, contains very small amounts of carotenoid or other pigment material and formation of a new pigment (echinochrome) in the early gastrula stage is readily observed. The striking appearance of pigment in early development suggests that the eggs of this animal might be unusually favorable material for a study of the cellular origin and chemo-differentiation of a defined substance—echinochrome. Echinochrome is a substituted naphthoquinone, red-purple in color, found in the test, spines, epidermis and various internal organs of sea urchins. The chemical structure and physical and chemical properties of certain echinochromes have been established by various investigators (Ball, 1936; Lederer and Glaser, 1938; Glaser and Lederer, 1939; Kuhn and Wallenfels, 1939, 1940; Wallenfels and Gauhe, 1943; Goodwin and Srisukh, 1950). A number of physiological functions have been ascribed to echinochrome. However, questions concerning the embryological and biochemical derivation, metabolism, and possible physiological functions of polyhydroxynaphthoquinones in echinoids are mainly unanswered.

The present study is an attempt to determine the embryonic origin of the echinochrome-forming cells, and to throw some light on the intracellular mechanisms affecting echinochrome synthesis.

MATERIALS AND METHODS

The eggs and sperm of the sea urchin *Lytechinus variegatus* were used throughout this work. *Arbacia punctulata* was used in some instances for comparison. The animals were collected in the Gulf of Mexico at the mouth of Alligator Harbor, Florida, using the facilities of the Florida State University Marine Laboratory. The animals were maintained in running sea water or jugs of continually aerated sea water at about 15 degrees centigrade.

The eggs and sperm were obtained from the animals by the KCl injection method. Eggs were fertilized in filtered sea water with approximately 0.5% sperm suspensions.

---

[1] Supported in part by a grant from the National Science Foundation to Dr. Charles B. Metz.

[2] Part of a thesis submitted to the graduate faculty of Florida State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] Food and Drug Administration, Bureau of Biological and Physical Sciences, Pharmacology Division, Washington, D. C.

Development was observed with the dissecting microscope and the phase contrast microscope. The ultra violet absorption spectrum of the echinochrome was determined by means of the Beckman DU Spectrophotometer.

All results are based on experiments involving at least 200 eggs. Each experiment was repeated at least twice. Any deviation from these numbers will be discussed in the text. The various techniques used in the individual experiments will be described with the results of the experiments for the sake of continuity and clarity.

RESULTS

Normal development

The fertilized egg of *Lytechinus* is relatively pigment-free. There is a very faint yellowish cast to the egg, probably due to carotenoids as in most echinoderm eggs, but the nucleus and cellular inclusions are clearly visible under the dissecting microscope.

The echinochrome-containing cells (echinophores) are first noticeable under the microscope during early gastrulation, when the veg_2 cell layer is invaginating to form the gut. They appear to be in the ectodermal layer and at first only in the region of the invagination. As gastrulation proceeds, the echinophores become dispersed throughout the gastrula, apparently in the ectodermal layer. In the pluteus, these cells are evenly dispersed in the outer body wall, with usually some concentration in the arm tips.

A series of experiments was designed to determine more precisely the origin of these cells in the course of development of the embryo and perhaps what intra- or inter-cellular mechanisms were involved.

Lithium experiments

Exogastrulae were produced using the technique of Herbst (1892) (treatment of fertilized eggs in a 0.1 *M* LiCl solution in sea water for five hours). This presented an opportunity to determine the effect of LiCl on pigmentation and to determine if the normal association of the germ layers is necessary for pigmentation. These exogastrulae were striking in that echinophores always appeared in the ectodermal portion of the larvae and never in the endoderm. The pigment appeared first at the site of evagination and by the time the evaginated gut was completely formed these cells were dispersed throughout the ectodermal wall of the larvae. The evaginated gut wall never contained pigment cells, although occasionally pigment could be seen inside the lumen where apparently it had migrated. This particular phenomenon will be discussed in more detail later, in connection with the amoeboid movements of these cells. From this experiment it may be said that pigment is associated with the ectoderm (at least in exogastrulae) and that a normal association of the germ layers is not necessary for pigment formation. LiCl in the concentrations (0.1 *M*–0.5 *M*) used had no apparent effect on pigmentation.

Thiocyanate experiments

Since the pigment cells were found only in the ectodermal portions of the LiCl-induced exogastrulae, it would be interesting to know if the ectoderm alone is able to give rise to these echinophores.

"Dauer" blastulae (permanent blastulae with no gut or skeleton) were formed using NaSCN (0.125 M) and the technique of Lindahl (1936). Those embryos which developed into "Dauer" blastulae were never pigmented. They remained as colorless hollow balls of ciliated ectodermal cells, whereas if there was any sign of invagination and appearance of endodermal derivatives, pigment was always formed in the ectodermal regions. In these cases, where very little endoderm was present, pigment cells were few and widely scattered. However, as in previous experiments, the pigment first appeared around the invaginating region, and later gradually became dispersed throughout the larval wall.

Apparently the ectoderm alone is not capable of giving rise to pigment, at least not in those "Dauer" blastulae formed by treatment with NaSCN.

Isolation experiments

It is possible to study the effects of ectodermization on pigmentation, without the influence of a chemical agent such as NaSCN. In these experiments, the technique of Hörstadius (1928) was used. The most satisfactory method for removing the fertilization membranes was found to be the shaking method of Driesch (1891).

Since *Lytechinus* eggs have no pigment band and removal of the fertilization membrane also removes the polar bodies at the animal pole, there is no sure way of determining which is the animal and which is the vegetal pole at the 8-cell stage. However, at the 16-cell stage, the micromeres have appeared and identification of the vegetal pole is quite easy. For this reason, most of the operations were done at the 16-cell stage. Some, however, were done at the 8-cell stage and will be discussed later. About 10 eggs at the 16-cell stage were placed in a drop of sea water in the shallow depression in the lid of a small stender dish, by means of a capillary pipette. The drop of sea water was kept as small as possible so that the surface tension served to hold the embryos in place while the cuts were being made. Under the dissecting microscope the desired blastomeres were separated with glass dissecting needles. The separated cells were picked up with the capillary pipette, transferred to sea water in syracuse dishes and allowed to develop.

By means of this technique, 30 animal and 30 vegetal halves were isolated at the 16-cell stage. Invariably, the animal halves formed typical "Dauer" blastulae, while the vegetal halves gastrulated and sometimes formed miniature plutei. These were usually defective in that they often had only one arm or a poorly formed gut and skeleton. The "Dauer" blastulae from isolated animal halves were never pigmented and remained unpigmented until they died. The vegetal half-embryos were always pigmented in a typical fashion.

Similar results were obtained in 40 cases in which halves of the 8-cell stage were isolated. However, since there was no way of identifying the animal and vegetal halves, the assumption was made that some of the cuts would isolate halves containing two animal and two vegetal cells, depending on the plane of the cut. This assumption appeared to be well founded, since some halves formed "Dauer" blastulae, while most developed into pigmented plutei. Only the animal halves form "Dauer" blastulae whereas the vegetal halves and those halves containing two animal and two vegetal cells gastrulate and develop pigment.

These experiments confirmed the results obtained in the NaSCN experiments, that is, that the ectoderm alone does not produce pigment. Pigment is formed when the embryo gastrulates (regardless of what fraction of the original egg is present) and only if the embryo gastrulates.

Vital staining experiments

In order to determine exactly what cells are responsible for the production of these echinophores, the technique of vital staining was employed. The technique of Hörstadius (1935) was used. The dye used was Grüblers "Neutral Rot," since this was the only dye found to penetrate the larvae satisfactorily.

The eggs were stained at the 16-cell stage. A fine glass capillary which was filled with agar containing a one per cent solution of neutral red was put into a drop of sea water in a stender dish lid. The egg to be stained was moved into position and held in place for about one minute, at which time enough dye was absorbed to render it readily identifiable. At the 16-cell stage the eight animal cells were stained in this way in a series of 12 eggs. These stained cells never became pigmented after gastrulation. The four micromeres in 12 eggs were stained in the same way and here, too, the stained cells never produced echinophores. When the four macromeres (of 12 eggs) were stained, the pigment cells in the pluteus showed traces of the dye. The macromeres do not divide until the end of the 32-cell stage, so that staining of the veg_1 and veg_2 cells derived from the macromeres is impossible until the 64-cell stage. At the time of the 64-cell stage, the individual blastomeres are extremely small and difficult to stain individually. However, an attempt was made to stain the cells comprising veg_1 in one series of eight eggs, and veg_2 in another series of eight eggs. It was found that the veg_2 cells and the micromere material always invaginated at gastrulation and gave rise to no pigmented cells. Although it was almost impossible to stain only veg_1 cells since some of the stain invariably got into veg_2 cells, it was none the less possible to see that it was from this material that the echinophores ultimately arose, since the concentration of the dye in the veg_1 cells was much greater. The material from veg_1 did not invaginate, but at the time of gastrulation, it remained near the site of invagination and ultimately gave rise to the ventral ectoderm of the pluteus.

Therefore, it would seem that the echinophores originate from the veg_1 cells but only under conditions permitting axial differentiation. The question then arises as to how these pigment cells become dispersed throughout the ectoderm of the pluteus, if their origin is localized in material which is ultimately destined to become only the ventral ectoderm of the pluteus. A series of observations gave an answer to this question.

Phase microscope observations

Boveri (1901) noticed amoeboid cells, of a brick red color, appearing in the late gastrula stage of the sea urchin *Paracentrotus lividus*. He considered the pigment the same as that in the egg. Monroy *et al.* (1951) also noted these amoeboid cells and suggested the possibility that the pigment in them might be echinochrome, although they were not able to obtain enough material to prove its presence. Neither of these workers reported a detailed study of the movements of the pigment cells, or their location.

These cells were studied in *Lytechinus variegatus* under the phase contrast microscope. In the early gastrulae when the echinophores first appeared, it was found that the cells were large, irregularly shaped structures in the region of the invagination. This would be the material derived from veg_1 cells. First, small pale orange pigment granules appeared. These were not easily visible except under high magnification. After a few hours, when the invagination process was almost complete, the pigment in these cells became darker red in color and much more concentrated, while the cells themselves increased greatly in number. At this time, it could be seen that the pigment cells were amoeboid in nature, apparently able to move freely within the ectodermal layer in any dimension. However, they were never seen to enter the endodermal layer underneath. By the pluteus stage, they had invaded the ectoderm of the larva and appeared to be most concentrated in the more actively growing arm tips of the pluteus. By the time the pluteus was fully formed, the echinophores had become much less motile and migration had virtually ceased.

In observations of eight exogastrulae, the same pattern was seen, except that the endoderm was no longer immediately under the ectoderm. The echinophores were seen occasionally to work themselves completely free of the ectoderm and come to lie in the cavity of the blastocoel and eventually even in the everted gut lumen. This explains the occasional appearance of pigment in the gut cavity of exogastrulae.

The next series of experiments was designed to determine what physical and chemical factors in the developing egg were responsible for the formation of the pigment and to obtain some information as to the relative roles played by the nucleus and cytoplasm in this synthesis. Since the pigment is elaborated long before the organism begins to take in food material from the outside, the pigment must be synthesized from pre-existing materials present in either the egg or sperm, or both.

Hybridization experiments

A series of experiments was designed to determine the effect of *Arbacia* × *Lytechinus* hybridization on subsequent pigmentation. Tennents' (1912) method (ageing of gametes for two hours followed by a five-minute treatment with alkaline sea water before fertilization) was used in obtaining these hybrids. The larvae from *Arbacia* male × *Lytechinus* female were all maternal in appearance. Pigmentation, size and general body structure of gastrulae (very few reached the pluteus stage) were typical of *Lytechinus*. The reverse cross, *Lytechinus* sperm × *Arbacia* egg, was unsuccessful in that no gastrulae were obtained and nothing could be seen concerning any newly formed pigment cells at gastrulation. These experiments were repeated three times with practically identical results.

It can be said only that *Arbacia* sperm cannot affect normal pigmentation in the

Lytechinus egg, in *Arbacia* sperm × *Lytechinus* egg hybrids. The question then arises as to the normal role, if any, played by the sperm in pigmentation.

One way of answering this question is to study the development of artificially activated eggs.

Parthenogenesis experiments

The (butyric acid-hypertonic sea water) method of Tennent (1912) was found to be the most satisfactory for producing parthenogenetic larvae. Again, it was noted that if gastrulation occurred, pigment was formed and apparently in the same fashion as described for normal fertilized eggs. The parthenogenetic plutei were normally pigmented and quite like the fertilized controls. These experiments were also repeated three times with the same results. It would seem that the presence of the sperm cell is not essential for pigment formation.

Identification of the pigment

The assumption has been made so far that the pigment in question is the substituted naphthoquinone, echinochrome. The proof of this lies in isolation, physical and chemical properties and spectrophotometric analysis. Echinochrome may be extracted by treatment with slightly acidified, organic solvents such as 80 per cent acetone or ether containing one per cent of HCl. The pigment may be then transferred by dilution into diethyl ether and chromatogrammed to remove impurities. The free compound shows very slight solubility in water or petroleum ether but is readily restored by shaking in air or by any of a number of mild oxidizing agents (Ball, 1936).

Clearly defined absorption bands are exhibited by solutions of echinochrome in various solvents. According to Kuhn and Wallenfels (1939), the absorption maxima of an echinochrome solution in carbon disulphide were 535, 499 and 464 mμ, in chloroform 532, 497 and 462 mμ, in benzene 532, 494 and 461 mμ and in concentrated sulfuric acid, 502 and 469 mμ.

The following evidence shows that the pigment appearing at gastrulation in the sea urchin, *Lytechinus variegatus,* has the properties of echinochrome.

(1) The pigment is orange-red in color.
(2) The pigment may be extracted from gastrulae and plutei by the above described procedure.
(3) The pigment, when extracted and dried, is nearly insoluble in water and petroleum ether.
(4) The pigment turns red in acid solution and violet in the alkaline range.
(5) The pigment is soluble in diethyl ether, acetone, ethanol and carbon disulfide.
(6) The pigment is very slightly soluble in chloroform.
(7) The addition of 5 mg. of sodium hydrosulfite to 10 ml. of a brick red solution of the pigment quickly bleaches the solution.
(8) The addition of small amounts of an oxidizing compound (hydrogen peroxide) or shaking in air quickly restores the color to the solution.
(9) The absorption spectrum of a carbon disulfide-pigment solution showed peaks on the Beckman DU Spectrophotometer at 530, 491, and 460 mμ.

Carotenoids, chromolipids, melanins and flavins may also be reddish in color (Sumner and Doudoroff, 1943). However, melanins and flavins (Mayer and Cook, 1943) are insoluble in almost all organic solvents. Flavins are water-soluble. The carotenoids may be bleached (Fox, 1936) but only by oxidizing agents rather than reducing agents. The absorption spectrum is typical of echinochrome and not carotenoids, since the carotenoid absorption peaks are around 510 and 485 mμ (Fox and Scheer, 1941). The change in color with changes in pH is also typical of quinone pigments. It was not possible to isolate and crystallize a sufficient amount of the pigment from plutei to permit further analysis of its physical and chemical properties but on the basis of the above evidence, the pigment appears to be echinochrome.

Chemo-differentiation study

The effect of a large number of inhibitors was studied in an attempt to specifically inhibit pigmentation and perhaps learn somethong of the metabolic pathway involved in its synthesis. It was found, however, that only those inhibitors which stopped development at gastrulation or ectodermized the eggs, such as 2-4-dinitrophenol, pyocyanine and iodosobenzoic acid, had an effect on pigmentation.

DISCUSSION

Boveri (1901) first noticed in the late gastrula stage of the sea urchin *Paracentrotus lividus* the appearance of amoeboid cells, rather heavily loaded with large pigment granules which differed from those of the unsegmented egg both with respect to their larger size and to their color. He noted that the number of these cells increased rapidly with the age of the embryo. Boveri, however, considered the pigment of the same nature as that of the egg. Monroy *et al.* (1951) noted that starting from the stage when the new pigment appeared, the embryos still retained a red-violet color after having been exhaustively extracted for carotenoids and that the remaining color was due to pigment still present in the amoeboid cells. These workers showed that the pigment could not be extracted with chloroform, acetone, methanol or pyridine but on slight acidification with dilute HCl, it could be taken up quantitatively in ether. It was thought the pigment was probably echinochrome but they were not able to obtain sufficient amounts to prove it spectrophotometrically. They also noted that the eggs of one female developed normally up to the beginning of gastrulation when exogastrulation occurred. In this case, they were unable to detect echinochrome. This observation seems unlikely in view of the exogastrulation experiments of the present study and was probably due to masking by other pigment.

Gustafson and Lenique (1951), using *Psammechinus miliaris,* mentioned pigment formation in the gastrula stage. They did not identify the pigment. However, they did mention that the red pigment cells became especially concentrated in the arm tips and the apical region, where the ectoderm is characterized by high mitochondrial activity. This observation is confirmed in the present study as previously mentioned, where echinophores are concentrated in the arm tips and apical region. It was also noted that in advanced starving plutei, the amount of echinochrome is appreciably reduced, suggesting the use of this protein-echinochrome complex as a food source under extreme conditions.

Using the *Lytechinus* egg in which the pigment is not sufficient to mask pigment elaboration subsequent to fertilization, it is possible to trace the differentiation of chromatophores with considerable accuracy. It was found that a pigment was synthesized in the embryo in the gastrula stage. This pigment was echinochrome. The particular cells in which the pigment appeared were shown by vital staining to originate from veg_1 and to be amoeboid in nature. With the use of isolation techniques and chemical treatment it was shown that pigment formation occurred only in association with gastrulation. Evidently, pigment cell differentiation is related to gastrulation in some way. Since the pigment cells differentiate in exogastrulae as well as in normal embryos, the differentiation does not appear to be an induction effect, at least to the extent that it requires the juxtaposition of endoderm with the other germ layers during gastrulation. It appears more likely that pigment cell differentiation, including the formation of pigment itself, is under the same control system as that governing the differentiation of other parts of the embryo (*e.g.,* skeleton, muscle, gut, coelom, etc.). From the work of Runnström (1933) and especially Hörstadius (1939) this over-all differentiation appears to depend upon the quantitative interaction of some sort of double gradient system. This system may function to produce pigment cells from veg_1 in normal development, but when the system is modified experimentally, for example by surgical or chemical treatments that result in "Dauer" blastulae, then pigment cells as well as other types of tissue fail to differentiate.

The production of echinochrome by the differentiating chromatophores presents an interesting problem in the chemo-differentiation of a defined substance. Unfortunately, no information is available concerning the pathways of echinochrome synthesis. However, echinochrome production may be correlated with protein synthesis, at least to the extent that new enzymes required for echinochrome synthesis may be elaborated by the embryo. Furthermore it is known that echinochrome occurs in the form of a protein complex (Kuhn and Wallenfels, 1940; Shapiro, 1946), and extensive protein synthesis begins at the same time that echinochrome first appears in the embryo, namely, at the time of gastrulation (Caspersson, 1947; Brachet, 1941; Zeuthen, 1951; Hultin, 1950; Perlmann, 1954). It is of interest to note the similarities between melanophore development in the vertebrates and echinophore development in the sea urchin. DuShane (1935) proved the neural crest origin of pigment cells in the amphibian. The formation of the neural crest and subsequent pigmentation are dependent on gastrulation in the amphibian and both amphibian and *Lytechinus* pigment cells are ectodermal in origin. In the amphibian, however, pigment cell formation is more complex, in that gastrulation induces the formation and differentiation of the neural crest, which in turn differentiates still further, giving rise to a number of structures, among which are the pigment cells. These cells, too, are amoeboid and migrate to their definitive position (Twitty and Niu, 1954) where they apparently lose their amoeboid capabilities and come to rest.

SUMMARY

1. A pigment having the properties of echinochrome is synthesized in the embryo of the sea urchin *Lytechinus variegatus*. Differentiation of the echinophores and synthesis of the echinochrome begins at the gastrula stage.

2. Echinophores differentiate from the veg_1 cell layer of the embryo, become amoeboid and migrate into other ectodermal regions.

3. Echinophore differentiation appears to depend upon a normal relation of the "double gradient" system of the embryo. Since echinophores were produced in exogastrulae, normal juxtaposition of the germ layers is not essential.

4. The sperm nucleus was found to have no essential role in the pigmentation process. Pigment formed according to the maternal pattern in hybrid and parthenogenetic embryos.

5. Of a variety of chemical substances tested, including several respiratory and other inhibitors, only those agents which inhibited gastrulation of the embryo caused failure of pigment formation.

6. Echinochrome synthesis is apparently related to protein synthesis in the embryo.

LITERATURE CITED

Ball, E. B., 1936. Echinochrome, its isolation and composition. *J. Biol. Chem.*, **114**: Proc. 30, vi.

Boveri, T., 1901. Die Polarität von Oocyte, Ei und Larve des *Strongylocentrotus lividus*. *Zool. Jahrb.*, **14**: 630–653.

Brachet, J., 1941. La detection histochimique et le microdosage des acides pentosenucleiques. *Enzymol.*, **10**: 87–98.

Caspersson, T., 1947. Relations between nucleic acid and protein synthesis. *Symp. Soc. Exp. Biol.*, **1**: 66–73.

Driesch, H., 1891. Entwickelungsmechanische Studien I. *Zeitschr. f. wiss. Zool.*, **53**: 600–637.

DuShane, G. P., 1935. An experimental study of the origin of pigment cells in Amphibia. *J. Exp. Zool.*, **72**: 1–31.

Fox, D. L., 1936. Structural and chemical aspects of animal coloration. *Amer. Nat.*, **70**: 477–493.

Fox, D. L., and B. T. Scheer, 1941. Comparative studies of the pigments of some Pacific coast echinoderms. *Biol. Bull.*, **80**: 441–455.

Glaser, R., and E. Lederer, 1939. Echinochrome et spinochrome; dérives methyles; distribution; pigments associés. *C. R. Acad. Sci., Paris,* **208**: 1939–1942.

Goodwin, T. W., and S. Srisukh, 1950. A study of the pigments of the sea urchins, *Echinus esculentus* and *Paracentrotus lividus*. *Biochem. J.*, **47**: 69–76.

Gustafson, T., and P. Lenique, 1951. Studies on mitochondria in the developing sea urchin egg. *Exp. Cell Res.*, **3**: 251–274.

Herbst, C., 1892. Experimentelle Untersuchungen über den Einfluss der veränderten chemischen Zusammensetzung des umgebenden Mediums auf die Entwicklung der Tiere. I. *Zeitschr. f. wiss. Zool.*, **55**: 446–518.

Hörstadius, S., 1928. Ueber die Determination des Keimes bei Echinodermen. *Acta Zool., Stockh.*, **9**: 1–33.

Hörstadius, S., 1935. Ueber die Determination im Verlaufe der Eiachse bei Seeigeln. *Pubbl. Staz. Zool. Napoli,* **14**: 251–478.

Hörstadius, S., 1939. The mechanics of sea urchin development, studied by operative methods. *Biol. Rev.*, **14**: 132–179.

Hultin, T., 1950. The protein metabolism of sea urchin eggs during early development studied by means of N-labeled ammonia. *Exp. Cell Res.*, **1**: 599–602.

Kuhn, R., and K. Wallenfels, 1939. Über die chemische Natur des Stoffes im Eier des Seeigels (*Arbacia pustulosa*) absondern, um die Spermatozoen anzulocken. *Berh. deutsch. chem. Ges.*, **72**: 1407–1413.

Kuhn, R., and K. Wallenfels, 1940. Echinochromes as prosthetic groups of high molecular symplexes in eggs of *Arbacia pustulosa*. *Chem. Abstr.*, **37**: 369–371.

Lederer, E., and R. Glaser, 1938. Sur l'echinochrome et le spinochrome. *C. R. Acad. Sci. Paris,* **207**: 454–456.

LINDAHL, P. E., 1936. Zur Kenntnis der physiologischen Grundlagen der Determination im Seeigelkeim. *Acta Zool., Stockh.,* **17**: 179–188.

MAYER, F., AND A. H. COOK, 1943. The Chemistry of Natural Coloring Matters. Rheinhold, New York. 273 p.

MONROY, A., A. M. ODDO AND M. DENICOLA, 1951. The carotenoid pigments during early development of the egg of the sea urchin *Paracentrotus lividus. Exp. Cell Res.,* **2**: 700–702.

PERLMANN, P., 1954. Study on the effect of antisera on unfertilized sea urchin eggs. *Exp. Cell Res.,* **6**: 485–490.

RUNNSTRÖM, J., 1933. Kunze Mitteilung zur Physiologie der Determination des Seeigelkeimes. *Arch f. Entw.,* **129**: 442–459.

SHAPIRO, H., 1946. The extracellular release of echinochrome. *J. Gen. Physiol.,* **29**: 267–275.

SUMNER, F. B., AND P. DOUDOROFF, 1943. An improved method of assaying melanin in fishes. *Biol. Bull.,* **84**: 187–194.

TENNENT, D. H., 1912. Studies in cytology. II. *J. Exp. Zool.,* **12**: 79–93.

TWITTY, V. C., AND M. C. NIU, 1954. The motivation of cell migration, studied by isolation of embryonic pigment cells singly and in small groups in vitro. *J. Exp. Zool.,* **125**: 541–574.

WALLENFELS, K., AND A. GAUHE, 1943. Synthesis of echinochrome. *Chem. Abstr.,* **37**: 5963.

ZEUTHEN, E., 1951. Segmentation, nuclear growth, and cytoplasmic storage in eggs of echinoderms and amphibia. *Pubbl. Staz. Zool. Napoli,* **23**: 47–69.

INDEX

ATP in crayfish tail muscle, 95.
Acclimation to temperature in goldfish tissues, 308.
Acorn barnacle, larval development of, 284.
Acoustical behavior of Bimini fishes, 357.
Acoustical orientation in bats, 10.
Activity of oysters at different temperatures, 57.
Actomyosin from crayfish tail muscle, 95.
Adaptation of goldfish tissues to high and low temperatures, 308.
Adenine, effect of on development of Ulva, 375.
Adenosine triphosphate in crayfish tail muscle, 95.
Adenylate kinase activity of crayfish tail muscle, 95.
Adult characteristics, assumption of by salamander efts, 1.
Agapema, cyclic carbon dioxide release in, 118.
Alga, marine, effect of plant hormones on, 375.
ALLEN, R. D., AND E. C. ROWE. The dependence of pigment granule migration on the cortical reaction in the eggs of Arbacia, 113.
Amicronucleate strain of Tetrahymena, DNA metabolism in, 71.
Amphibian, water drive in, 1.
Anaerobic capacity of Cecropia pupal heart, 23.
Anatomy of Balanus larvae, 284.
Anatomy of stomatopods, 141.
Apyrase activity of crayfish tail muscle, 95.
Arbacia, development of pigment in larvae of, 394.
Arbacia eggs, cortical reaction in, 113.
Arbacia eggs, x-irradiation of, 385.
Arbacia plutei as food for Balanus larvae, 284.
Attachment of Bugula larvae, 215.
Auditory phenomena in bats, 10.
Auditory phenomena in fishes, 357.
Autoradiography of sea urchin eggs, 196.

BACTERIA, role of in inhibition of regeneration in Tubularia, 255.
Bacteria-free cultures of Ulva, 375.
Bahamian fishes, acoustical behavior of, 357.
Balanus, larval development of, 284.
Barnacle, larval development of, 284.
Barnacle ova, fungus parasite in, 205.
Bats, echolocation in, 10.
Behavior, acoustical, of Bimini fishes, 357.
Behavior of oysters at different temperatures, 57.
Beta-mercaptoethylamine, "protective" effects of against x-irradiation damage to Arbacia eggs, 385.
Bimini fishes, acoustical behavior of, 357.
Bioassay of prolactin, 1.
Biology of five-lunuled sand dollar, 54.
Biology of oyster crab, 146.
Bivalve, effect of temperature on behavior of, 57.
Blastomere interaction in Dendraster, 247.
Blockage to development in frogs' eggs, 226.
Blood analyses of hagfish, 348.
Blood sodium and potassium in Pachygrapsus, 334.
BOOKHOUT, C. G. *See* J. D. COSTLOW, JR., 284.
BOROUGHS, H. *See* S. C. HSIAO, 196.
Brachiopoda, chitin in, 106.
Brain respiration of goldfish, 308.
Bryozoa, chitin in, 106.
Bryozoan larvae, rate of attachment of, 215.
BUCK, J. Cyclic carbon dioxide release in insects. IV., 118.
Bugula larvae, rate of attachment of, 215.
Burrowing activities of Mellita, 54.
BRYAN, J. H. D., AND J. W. GOWEN. The effects of 2560 r of x-rays on spermatogenesis in the mouse, 271.

CALCIUM, radioactive, uptake of by sea urchin eggs, 196.
Calcium, role of in Dendraster twinning, 247.
Cambarellus, chromatophorotropins of, 317.
Cambarus, contractile protein from muscle of, 95.
Cape Cod, new stomatopod from, 141.
Carbon dioxide release by oxygen-treated Habrobracon white pupae, 180.
Carbon dioxide release in insects, 118.
Carbon monoxide sensitivity of silkworm pupae, 36.
Cecropia, cyclic carbon dioxide release by, 118.
Cecropia, diapause in, 23, 36.
Cell division in mouse testis, effects of x-rays on, 271.

Cell division in x-irradiated Arbacia eggs, 385.
CHACE, F. A., JR. A new stomatopod crustacean of the genus Lysiosquilla from Cape Cod, Mass., 141.
Chitin in lophophorate phyla, 106.
Chlamydomonas as food for Balanus larvae, 284.
Chloride concentrations in hagfish blood, 348.
CHRISTENSEN, A. M., AND J. J. MCDERMOTT. Life-history and biology of the oyster crab, Pinnotheres, 146.
Chromatophorotropins of crayfishes, 317.
Chthamalus ova, fungus parasite in, 205.
Cirripede, larval development of, 284.
Clam larvae, survival and growth of at different salinities, 296.
CLARK, A. M., AND M. J. PAPA. Some effects of oxygen upon the white pupae of Habrobracon, 180.
Cleavage of mercaptoethanol-treated Dendraster eggs, 247.
Cleavage of x-irradiated Arbacia eggs, 385.
Coelenterate, inhibition of regeneration in, 255.
Cold, effect of on behavior of oysters, 57.
Cold, effect of on development of Urosalpinx, 188.
Cold, effect of on eclosion rate of Habrobracon white pupae, 180.
Cold, effect of on goldfish tissue respiration, 308.
Cold, role of in oxygen-poisoning of frogs' eggs, 226.
Color changes in crayfishes, 317.
Contractile protein from crayfish tail muscle, 95.
Copulation in Pinnotheres, 146.
Cortical reaction in Arbacia eggs, 113.
COSTLOW, J. D., JR., AND C. G. BOOKHOUT. Larval development of B. amphitrite var. denticulata reared in the laboratory, 284.
Crab, ion regulation in, 334.
Crab, oyster, life-history of, 146.
Crassostrea, effect of temperature on, 57.
Crassostrea, pea crab from, 146.
Crassostrea larvae, survival and growth of at different salinities, 296.
Crayfish tail muscle, contractile protein from, 95.
Crowding, role of in Tubularia regeneration, 255.
Crustacean, ion regulation in, 334.
Crustacean, life-history of, 146.
Crustacean, stomatopod, from Cape Cod, 141.
Crustacean chromatophorotropins, 317.
Crustacean eggs, fungus parasite of, 205.
Crustacean muscle, contractile protein from, 95.
Crustacean tissues, enzyme activity of, 95.
Cyanide-sensitivity of Cecropia pupal heartbeat, 23.
Cyclic carbon dioxide release in insects, 118.
Cyprid larvae of Balanus, 284.
Cysteinamine, "protective" effects of against x-irradiation damage to Arbacia eggs, 385.
Cytochemistry of Tetrahymena, 71.
Cytochrome system in Cecropia pupa, 23, 36.
Cytoplasm, egg, uptake of radiocalcium by, 196.
Cytoplasmic granules in Arbacia egg, 113.

DNA metabolism in Tetrahymena, 71.
DNA synthesis in mouse testis, effects of x-rays on, 271.
Darkness, role of in development of Ulva, 375.
"Dauerblastulae" of Lytechinus, 394.
DAVIS, H. C. Survival and growth of clam and oyster larvae at different salinities, 296.
Desiccation, effects of on Pachygrapsus, 334.
Dendraster, twinning in, 247.
Desoxyribonucleic acid metabolism of Tetrahymena, 71.
Detergent, effect of on radiocalcium uptake of sea urchin eggs, 196.
Development of Balanus, 284.
Development of bivalve larvae in relation to salinity, 296.
Development of Dendraster twins, 247.
Development of Habrobracon pupae, 180.
Development of oxygen-poisoned frogs' eggs, 226.
Development of parasitized barnacle eggs, 205.
Development of pigment in Lytechinus, 394.
Development of Pinnotheres, 146.
Development of sperm in mouse testis, effects of x-rays on, 271.
Development of Ulva, effect of plant hormones on, 375.
Development of Urosalpinx, 188.
Development of x-irradiated Arbacia eggs, 385.
Diapause, insect, physiology of, 23, 36.
Dichlorobenzenonindophenol, effect of on attachment of Bugula larvae, 215.
Diemyctylus efts, water drive studies on, 1.
Differentiation in regenerating Tubularia, 255.
Differentiation of x-irradiated Bugula larvae, 215.
Diffusion of gases in insects, 118.
Distribution of fishes in Bimini area, 357.
Division, cell, in Dendraster, blockage of by mercaptoethanol, 247.
Division of Tetrahymena, 71.
Drill, oyster, development of, 188.
Dwarf crayfish, chromatophorotropins of, 317.

ECHINOCHROME migration in Arbacia eggs, 113.
Echinoderm, pigment development in larva of, 394.
Echinoderm eggs, cortical reaction in, 113.
Echinoderm eggs, twinning in, 247.
Echinoderm eggs, uptake of radiocalcium by, 196.
Echinoid, biology of, 54.
Echolocation in bats, 10.
Eclosion of Habrobracon white pupae, 180.
Ecological factors in relation to behavior of oysters, 57.
Ecology of Chthamalus in relation to fungus parasite, 205.
Ecology of Pinnotheres, 146.
Ecology of Urosalpinx, 188.
Ectoprocta, chitin in, 106.
Effect of plant hormones on Ulva, 375.
Effect of x-irradiation on Bugula attachment, 215.
Effects of oxygen on Habrobracon white pupae, 180.
Effects of x-rays on mouse testis, 271.
Efts, water drive studies on, 1.
Egg-deposition of Pinnotheres, 146.
Eggs, Arbacia, cortical reaction in, 113.
Eggs, Arbacia, x-irradiation of, 385.
Eggs, barnacle, fungus parasite in, 205.
Eggs, frog, oxygen poisoning of, 226.
Eggs, sea urchin, uptake of radiocalcium by, 196.
Eggs, Urosalpinx, development of, 188.
EKBERG, D. R. Respiration in tissues of goldfish adapted to high and low temperatures, 308.
Electrolytes in hagfish blood, 348.
Embryo, sea urchin, development of pigment in, 394.
Embryology of mercaptoethanol-treated Dendraster eggs, 247.
Embryology of parasitized barnacle eggs, 205.
Embryology of Urosalpinx, 188.
Embryology of x-irradiated Arbacia eggs, 385.
Embryos, frog, oxygen poisoning of, 226.
Embryos, twin, in Dendraster, 247.
Endocrine studies on salamander efts, 1.
Enzyme kinetics in crayfish muscle extracts, 95.
Epinephalus, acoustical behavior of, 357.
Epinephrine, effect of on red chromatophorotropins of crayfish, 317.
Erdschreiber medium for culture of Ulva, 375.
Evolution of telson in stomatopods, 141.
Evolutionary significance of hagfish osmotic properties, 348.
Exogastrulation in Lytechinus eggs, 394.

FEEDING of Mellita, 54.
Feeding of oysters at different temperatures, 57.
Fertilization of Arbacia eggs before x-irradiation, 385.
Fertilization reaction in Arbacia eggs, 113.
FINGERMAN, M., AND M. E. LOWE. Stability of the chromatophorotropins of the dwarf crayfish, Cambarellus, and their effects on another crayfish, 317.
Fishes, Bimini, acoustical behavior of, 357.
Five-lunuled sand dollar, biology of, 54.
Flow of water through oysters at different temperatures, 57.
Flowing gases, effects of on silkworm heartbeat, 23, 36.
Food in relation to survival of bivalve larvae at different salinities, 296.
Freezing-point measurements of hagfish blood, 348.
Frogs' eggs, oxygen poisoning of, 226.
Fungus parasite in barnacle ova, 205.

GAMETES, methods for obtaining, from clams and oysters, 296.
GANAROS, A. E. On development of early stages of Urosalpinx at constant temperatures and their tolerance to low temperatures, 188.
Gas exchange in insects, 118.
Gases, effects of on silkworm pupal heartbeat, 23, 36.
Gastrular blockage in frogs' eggs, 226.
Gibberellin, effect of on development of Ulva, 375.
Gill oxygen consumption of goldfish, 308.
Goldfish tissues, respiration in, 308.
Gonad, mouse, effects of x-rays on, 271.
Gonadotropin, role of in water drive of salamander eft, 1.
GOWEN, J. W. *See* J. H. D. BRYAN, 271.
GRANT, W. C., JR., AND J. A. GRANT. Water drive studies on hypophysectomized efts of Diemyctylus. I., 1.
Granule migration in Arbacia egg cortex, 113.
GRIFFIN, D. R. *See* A. D. GRINNELL, 10.
GRINNELL, A. D., AND D. R. GRIFFIN. The sensitivity of echolocation in bats, 10.
GROSS, W. J. Potassium and sodium regulation in an intertidal crab, 334.
Grouper, acoustical behavior of, 357.
Growth cycle of Tetrahymena, 71.
Growth of bivalve larvae at different salinities, 296.
Growth of Pinnotheres, 146.
Growth of regenerating Tubularia, 255.

Growth of Ulva germlings, 375.
Growth of x-irradiated Bugula larvae, 215.

HABROBRACON, effects of oxygen on white pupae of, 180.
Hagfish, osmotic properties of, 348.
HARVEY, W. R., AND C. M. WILLIAMS. Physiology of insect diapause. XI., XII., 23, 36.
Hatching of Pinnotheres, 146.
Hawaiian sea urchin, uptake of radiocalcium by eggs of, 196.
Heartbeat of Cecropia pupa, 23, 36.
Heat, effect of on development of Urosalpinx, 188.
Heat, effect of on eclosion of Habrobracon white pupae, 180.
Heat, effect of on goldfish tissue respiration, 308.
High temperature, effect of on behavior of oysters, 57.
High temperature, effect of on goldfish tissue respiration, 308.
Histochemistry of chitin in lophophorate phyla, 106.
Holocentrus, acoustical behavior of, 357.
Hormone, lactogenic, role of in water drive of efts, 1.
Hormones, chromatophorotropic, in crayfish, 317.
Hormones, plant, effect of on development of Ulva, 375.
Host size in relation to growth and development of Pinnotheres, 146.
HSIAO, S. C., AND H. BOROUGHS. The uptake of radiocalcium by sea urchin eggs. I., 196.
Hyalophora, cyclic carbon dioxide release in, 118.
Hybridization of Lytechinus and Arbacia, 394.
Hydrogen peroxide, role of in attachment of Bugula larvae, 215.
HYMAN, L. H. Notes on the biology of the five-lunuled sand dollar, 54.
HYMAN, L. H. The occurrence of chitin in the lophophorate phyla, 106.
Hypophysectomized efts, water drive studies on, 1.

IAA, effect of on development of Ulva, 375.
IAA, effect of on goldfish tissue respiration, 308.
Indolacetic acid, effect of on development of Ulva, 375.
Iodoacetic acid, effect of on goldfish tissue respiration, 308.
Infection of barnacle eggs with fungus parasite, 205.
Infection of oysters with Pinnotheres, 146.
Inhibitors of regeneration in Tubularia, 255.
Insect diapause, physiology of, 23, 36.
Insects, cyclic carbon dioxide release in, 118.
Insemination reaction in Arbacia eggs, 113.
Instars of Pinnotheres, 146.
Intertidal crab, sodium and potassium regulation in, 334.
Ion regulation in crab, 334.
Ionizing radiations, effect of on attachment of Bugula larvae, 215.
Ionizing radiations, effect of on sea urchin eggs, 385.
Irradiation, roentgen, of Arbacia egg, 385.
Irradiation, roentgen, of Bugula larvae, 215.
Isolation, "chemical," of Dendraster blastomeres, 247.
Isolation of animal and vegetal halves of Lytechinus embryos, 394.
Isotope, radioactive, uptake of by sea urchin eggs, 196.

JELLY coat of sea urchin egg, role of in radiocalcium uptake, 196.
JOHNSON, T. W., JR. A fungus parasite in ova of the barnacle Chthamalus, 205.

KEY to stomatopods, 141.
Kinetics of enzyme action in crayfish tail muscle extracts, 95.
Kinetin, effect of on development of Ulva, 375.

LABORATORY culture of Balanus, 284.
Lactogenic hormone, role of in water drive of efts, 1.
Lagenidium as parasite of barnacle eggs, 205.
Larva of Lytechinus, pigment development in, 394.
Larvae, bivalve, survival and growth of at different salinities, 296.
Larvae, Bugula, rate of attachment of, 215.
Larval development of Balanus, 284.
Larval stages of Pinnotheres, 146.
Lethal effects of carbon monoxide on silkworm pupae, 36.
Lethal effects of cyanide on silkworm pupae, 23.
Life-history of Balanus, 284.
Life-history of oyster crab, 146.
Light, role of in development of Ulva, 375.
Light-reversible inhibition by carbon monoxide in silkworm pupae, 36.
Lithium-treatment of Lytechinus eggs, 394.
Liver respiration of goldfish, 308.

Loosanoff, V. L. Some aspects of behavior of oysters at different temperatures, 57.
Lophophorate phyla, chitin in, 106.
Low temperature, effect of on behavior of oysters, 57.
Low temperature, effect of on development of Urosalpinx, 188.
Low temperature, effect of on eclosion rate of Habrobracon white pupae, 180.
Low temperature, effect of on goldfish tissue respiration, 308.
Low temperature, role of in oxygen poisoning of frogs' eggs, 226.
Lowe, M. E. *See* M. Fingerman, 317.
Lunules of Mellita, 54.
Lynch, W. F. The effect of x-rays, irradiated sea water, and oxidizing agents on the rate of attachment of Bugula larvae, 215.
Lysiosquilla, new species of, 141.
Lytechinus, development of pigment in larvae of, 394.

MACRONUCLEAR division in Tetrahymena, 71.
Malamed, S. Gastrular blockage in frogs' eggs produced by oxygen poisoning, 226.
Mammalian testis, effects of x-rays on, 271.
Marine alga, effect of plant hormones on, 375.
Marine bryozoa, attachment of, 215.
Marine eggs, fungus parasite in, 205.
Marine fishes, sound production by, 357.
Marine hagfishes, osmotic properties of, 348.
Maruyama, K. Contractile protein from crayfish tail muscle, 95.
Maturation of mouse sperm, effects of x-rays on, 271.
Mazia, D. The production of twin embryos in Dendraster by means of mercaptoethanol, 247.
McDermott, J. J. *See* A. M. Christensen, 146.
McDonald, B. B. Quantitative aspects of DNA metabolism in an amicronucleate strain of Tetrahymena, 71.
McFarland, W. N., and F. W. Munz. A re-examination of the osmotic properties of the Pacific hagfish, Polistotrema, 348.
Mechanism of cyclic carbon dioxide release in insects, 118.
Meiosis, effects of x-rays on, in mouse testis, 271.
Mellita, biology of, 54.
Mercaptoethanol, use of in producing Dendraster twins, 247.
Metabolism of Cecropia diapause, 23, 36.
Metabolism of DNA in Tetrahymena, 71.
Metabolism of goldfish tissues, 308.
Metabolism of Habrobracon white pupae, 180.
Metabolism of insects, 118.
Metamorphosis of Bugula larvae, effects of various agents on, 215.
Metamorphosis of silkworm pupae, 23, 36.
Migration of pigment granules in Arbacia eggs, 113.
Migration of salamander efts to water, 1.
Mitosis, effects of x-rays on, in mouse testis, 271.
Mitosis in Dendraster eggs, blockage of by mercaptoethanol, 247.
Mitosis in x-irradiated Arbacia eggs, 385.
Mollusc, behavior of at different temperatures, 57.
Mollusc larvae, survival and growth of at different salinities, 296.
Molting of hypophysectomized salamander efts, 1.
Molting stages of Pinnotheres, 146.
Monothioethylene glycol, use of in producing Dendraster twins, 247.
Morphogenesis of Ulva, effect of plant hormones on, 375.
Morphogenesis of x-irradiated Arbacia eggs, 385.
Morphology of Balanus larvae, 284.
Morphology of barnacle egg fungus parasite, 205.
Morphology of Pinnotheres developmental stages, 146.
Morphology of stomatopods, 141.
Moulton, J. M. The acoustical behavior of some fishes in the Bimini area, 357.
Mouse, effects of x-rays on spermatogenesis in, 271.
Movement of oyster shells at different temperatures, 57.
Munz, F. W. *See* W. N. McFarland, 348.
Muscle, crayfish, contractile protein from, 95.
Myosin in crayfish tail muscle, 95.
Myotis, sound emission by, 10.
Myxinoid, osmotic properties of, 348.

NAUPLII of Balanus, 284.
Neurosecretory system of dwarf crayfish, 317.
New stomatopod from Cape Cod, 141.
Nitrogen treatment of frog eggs, 226.
Nucleic acid metabolism of Tetrahymena, 71.

OCCURRENCE of chitin in lophophorate phyla, 106.
Orconectes, effect of Cambarellus chromatophorotropins on, 317.
Orientation in bats, 10.
Origin of pigment in Lytechinus, 394.

Osmotic properties of hagfish, 348.
Osmotic regulation in Pachygrapsus, 334.
Ova, Arbacia, cortical reaction in, 113.
Ova, Arbacia, x-irradiation of, 385.
Ova, barnacle, fungus parasite in, 205.
Ova, Dendraster, treatment of with mercaptoethanol, 247.
Ova, frog, oxygen poisoning of, 226.
Ova, sea urchin, uptake of radiocalcium by, 196.
Ova, Urosalpinx, development of, 188.
Oxidizing agents, effect of on rate of attachment of Bugula larvae, 215.
Oxygen, effect of on silkworm pupal heart, 23, 36.
Oxygen, effects of on Habrobracon white pupae, 180.
Oxygen poisoning of frogs' eggs, 226.
Oxygen uptake of goldfish tissues, 308.
Oxygen uptake in insects, 118.
Oyster crab, life-history of, 146.
Oyster drill, development of, 188.
Oyster larvae, survival and growth of at different salinities, 296.
Oysters, behavior of at different temperatures, 57.

PACHYGRAPSUS, ion regulation in, 334.
Pacific hagfish, osmotic properties of, 348.
PAPA, M. J. *See* A. M. CLARK, 180.
Parasite, fungus, in barnacle ova, 205.
Parasitic wasp pupae, effects of oxygen on, 180.
Parthenogenesis in Lytechinus, 394.
Partial fertilization of Arbacia eggs, 113.
Pea crab, life-history of, 146.
Peroxide, role of in attachment of Bugula larvae, 215.
Phoronida, chitin in, 106.
Phosphorus in crayfish tail muscle, 95.
Photo-reversal of CO-inhibition in silkworm pupae, 36.
Phycomycete parasite of barnacle eggs, 205.
Phylogenetic significance of chitin, 106.
Physiology of insect diapause, 23, 36.
Pigment-concentrating and -dispersing hormones in crayfishes, 317.
Pigment-development in Lytechinus, 394.
Pigment granule migration in Arbacia eggs, 113.
Pigmentation, adult, assumption of by salamander efts, 1.
Pigmentation of developing Ulva, effect of plant hormones on, 375.
Pigmentation of Habrobracon pupae, effects of oxygen on, 180.
Pinnotheres, life-history of, 146.
Pituitary hormones, role of in eft water drive, 1.
Plant hormones, effect of on development of Ulva, 375.
Platysamia, physiology of insect diapause in, 23, 36.
Podia of Mellita, 54.
Polistotrema, osmotic properties of, 348.
Polyphemus, physiology of diapause in, 23, 36.
Population differences in survival of bivalve larvae at different salinities, 296.
Potassium regulation in crab, 334.
Pressure, effects of on development of frog eggs, 226.
Pressure, effects of on Habrobracon white pupae, 180.
Production of Dendraster twins, 247.
Prolactin, role of in water drive of efts, 1.
Protease, use of in removing Dendraster fertilization membrane, 247.
"Protection" against x-irradiation effects in Arbacia eggs, 385.
Protein, contractile, from crayfish tail muscle, 95.
Protozoan, DNA metabolism in, 71.
PROVASOLI, L. Effect of plant hormones on Ulva, 375.
Pumping activity of oysters at different temperatures, 57.
Pupae of Habrobracon, effects of oxygen on, 180.
Pupal diapause, physiology of, 23, 36.

QUANTITATIVE aspects of DNA metabolism in Tetrahymena, 71.

RADIATION effects on Arbacia egg, 385.
Radiation-sensitive constituents of mouse testis, 271.
Radiocalcium, uptake of by sea urchin eggs, 196.
Radiosensitivity of Arbacia eggs, 385.
Rana eggs, oxygen poisoning of, 226.
Rate of attachment of Bugula larvae, 215.
Rate of development of oxygen-poisoned frog eggs, 226.
Recognition behavior in black angelfish, 357.
Recording of fish acoustical behavior, 357.
"Recovery" phenomenon in x-irradiated Arbacia eggs, 385.
Red efts, water drive of, 1.
Reducing agents, effects of on Bugula larvae, 215.
Re-examination of hagfish osmotic properties, 348.
Regeneration inhibitors in Tubularia, 255.

Regulation of sodium and potassium in crab, 334.
Release of carbon dioxide in insects, 118.
Reproductive potential of bivalves in relation to salinity, 296.
Respiration of Habrobracon white pupae, 180.
Respiration in insects, 118.
Respiration in tissues of goldfish, 308.
Roentgen irradiation of Arbacia eggs, 385.
Roentgen irradiation of Bugula larvae, 215.
Roentgen irradiation of mouse testis, 271.
ROWE, E. C. *See* R. D. ALLEN, 113.
RUGH, R. The so-called "recovery" phenomenon and "protection" against x-irradiation at the cellular level, 385.

SALAMANDER eft, water drive in, 1.
Salinity in relation to survival and growth of bivalve larvae, 296.
"Salt pools" in Pachygrapsus, 334.
Sand dollar, five-lunuled, biology of, 54.
Sea urchin eggs, cortical reaction in, 113.
Sea urchin eggs, twinning in, 247.
Sea urchin eggs, uptake of radiocalcium by, 196.
Sea urchin eggs, x-irradiation of, 385.
Sea urchin larvae, pigment development in, 394.
Sea water, irradiated, effect of on attachment of Bugula larvae, 215.
Sea weed, effect of plant hormones on, 375.
Seasonal variations in goldfish oxygen consumption, 308.
Seminiferous tubules of mouse, effects of x-rays on, 271.
Sensitivity of echolocation in bats, 10.
Sensitivity of Habrobracon pupae to oxygen, 180.
Serum chloride and sodium concentrations of hagfish blood, 348.
Setation formulae of larval barnacles, 284.
Setting of x-irradiated Bugula larvae, 215.
Sexual reproduction in fungus parasite of barnacle egg, 205.
Shaking, role of in oxygen poisoning of frog eggs, 226.
Shell-opening of oysters at different temperatures, 57.
Shore crab, ion regulation in, 334.
Silkworm, diapause in, 23, 36.
Slime, role of in osmotic properties of hagfish blood, 348.
Sodium concentrations in hagfish blood, 348.
Sodium regulation in crab, 334.
Sonic experiments with Bimini fishes, 357.
Sound emission by bats, 10.
Spat, oyster, infection of with Pinnotheres, 146.
Spermatogenesis in mouse, effects of x-rays on, 271.
Spiracles, role of in cyclic release of carbon dioxide by insects, 118.
Spontaneous production of sound by Bimini fishes, 357.
Squirrelfish, acoustical behavior of, 357.
Stability of crayfish chromatophorotropins, 317.
Stomatopod from Cape Cod, 141.
Stress, osmotic, in crab, 334.
Sulfhydryl bond, possible role of in oxygen poisoning of frog embryos, 226.
Sulfhydryl bond, possible role of in x-irradiation of Bugula larvae, 215.
Sulfhydryl group, importance of in x-irradiated Arbacia eggs, 385.
Sulfhydryl groups, importance of in Dendraster twinning, 247.
Survival of bivalve larvae at different salinities, 296.
Synthesis of DNA in Tetrahymena, 71.

TTC, effect of on attachment of Bugula larvae, 215.
Taxonomy of stomatopods, 141.
Telea, physiology of diapause of, 23, 36.
Teleost, fresh-water, respiration of tissues of, 308.
Teleosts, sound production by, 357.
Temperature, effect of in cyanide-sensitivity of silkworm pupae, 23.
Temperature, effect of on behavior of oysters, 57.
Temperature, effect of on goldfish tissue respiration, 308.
Temperature, effect of on pigment concentration in crayfish, 317.
Temperature, relation of to development of Urosalpinx, 188.
Temperature, relation of to eclosion of Habrobracon white pupae, 180.
Temperature, role of in oxygen-poisoning of frogs' eggs, 226.
Terrestrial stage of Diemyctylus, water drive in, 1.
Tetrahymena, DNA metabolism in, 71.
Thiol groups, importance of in Dendraster twinning, 247.
Tissue culture of Ulva, 375.
Tissue extracts of Tubularia as inhibiting agents, 255.
Tissues of goldfish, respiration in, 308.
Toothplate stridulation in marine fishes, 357.
Triphenyltetrazolium chloride, effect of on attachment of Bugula larvae, 215.
Tripneustes, uptake of radiocalcium by eggs of, 196.

Triturus, water drive of, 1.
Tropical fishes, acoustical behavior of, 357.
Tube feet of Mellita, 54.
Tubularia, inhibition of regeneration in, 255.
TWEEDELL, K. Inhibitors of regeneration in Tubularia, 255.
Twinning in Dendraster, 247.

ULTRAVIOLET absorption spectra of crayfish tail muscle extracts, 95.
Ulva, effect of plant hormones on, 375.
Underwater sounds in Bimini area, 357.
Unfertilized sea urchin eggs, uptake of radiocalcium by, 196.
Uptake of radiocalcium by unfertilized sea urchin eggs, 196.

VENUS larvae, survival and growth of, at different salinities, 296.
Vital staining of Lytechinus embryos, 394.

WARM-ADAPTED goldfish tissues, respiration of, 308.
Wasp pupae, effects of oxygen on, 180.
Water drive studies on hypophysectomized efts, 1.
Water flow through oysters at different temperatures, 57.
WILLIAMS, C. M. *See* W. R. HARVEY, 23, 36.

X-IRRADIATION of Arbacia eggs, 385.
X-irradiation of Bugula larvae, 215.
X-rays, effects of on mouse testis, 271.

YOUNG, R. S. Development of pigment in the larva of the sea urchin, Lytechinus, 394.

ZOID metamorphosis after x-irradiation, 215.

Volume 114 Number 1

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

HAROLD C. BOLD, University of Texas
FRANK A. BROWN, JR., Northwestern University
JOHN B. BUCK, National Institutes of Health
T. H. BULLOCK, University of California, Los Angeles
E. G. BUTLER, Princeton University
J. H. LOCHHEAD, University of Vermont
ARTHUR W. POLLISTER, Columbia University
C. L. PROSSER, University of Illinois
MARY E. RAWLES, Carnegie Institution of Washington
WM. RANDOLPH TAYLOR, University of Michigan
A. R. WHITING, University of Pennsylvania
CARROLL M. WILLIAMS, Harvard University

DONALD P. COSTELLO, University of North Carolina
Managing Editor

Marine Biological Laborat
LIBRARY
MAR 1 4 1958
WOODS HOLE, MASS.

FEBRUARY, 1958

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

INSTRUCTIONS TO AUTHORS

The Biological Bulletin accepts papers on a variety of subjects of biological interest. In general, however, review papers (except those written at the specific invitation of the Editorial Board), short preliminary notes and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within three months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before they are refereed by the Board.

1. *Manuscripts.* Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided unless they are absolutely necessary, and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. A *condensed title* or running head of no more than 35 letters and spaces should be included.

4. *Literature Cited.* The list of papers cited should conform **exactly** to the style set in a recent issue of The Biological Bulletin; this list should be headed LITERATURE CITED, and typed *double*-spaced on separate pages.

5. *Figures.* The dimensions of the printed page, 5 by 7⅝ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction. Explanatory matter should be included in legends as far as possible, not lettered on the illustrations. Figures should be prepared for reproduction as line cuts or halftones; other methods will be used only at the author's expense. Figures to be reproduced as line cuts should be drawn in black ink on white paper, good quality tracing cloth or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on Bristol Board, and any designating numbers or letters should be made directly on the figures. *All* figures should be numbered in consecutive order, with no distinction between text- and plate-figures. The author's name should appear on the reverse side of all figures, as well as the desired reduction.

6. *Mailing.* Manuscripts should be packed flat; large illustrations may be rolled in a mailing tube. **All illustrations larger than 8½ by 11 inches must be accompanied by photographic reproductions or tracings that may be folded to page size.**

Reprints. Authors will be furnished, free of charge, one hundred reprints without covers. Additional copies may be obtained at cost; approximate prices will be furnished by the Managing Editor upon request.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 1 and September 1, and to Dr. Donald P. Costello, P. O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa.
under the Act of August 24, 1912.

BIOLOGY MATERIALS

The Supply Department of the Marine Biological Laboratory has a complete stock of excellent plain preserved and injected materials, and would be pleased to quote prices on school needs.

* * *

PRESERVED SPECIMENS

for

Zoology, Botany, Embryology,
and Comparative Anatomy

LIVING SPECIMENS

for

Zoology and Botany

including Protozoan and Drosophila Cultures, and Animals for Experimental and Laboratory Use.

MICROSCOPE SLIDES

for

Zoology, Botany, Embryology, Histology, Bacteriology, and Parasitology.

CATALOGUES SENT ON REQUEST

* * *

Supply Department

MARINE
BIOLOGICAL LABORATORY
Woods Hole, Massachusetts

CONTENTS

Page

GRANT, WILLIAM C., JR., AND JOAN A. GRANT
Water drive studies on hypophysectomized efts of Diemyctylus viridescens 1

GRINNELL, ALAN D., AND DONALD R. GRIFFIN
The sensitivity of echolocation in bats 10

HARVEY, WILLIAM R., AND CARROLL M. WILLIAMS
Physiology of insect diapause. XI. Cyanide-sensitivity of the heartbeat of the Cecropia silkworm, with special reference to the anaerobic capacity of the heart 23

HARVEY, WILLIAM R., AND CARROLL M. WILLIAMS
Physiology of insect diapause. XII. The mechanism of carbon monoxide-sensitivity and -insensitivity during the pupal diapause of the Cecropia silkworm 36

HYMAN, LIBBIE H.
Notes on the biology of the five-lunuled sand dollar 54

LOOSANOFF, V. L.
Some aspects of behavior of oysters at different temperatures 57

MCDONALD, BARBARA BROWN
Quantitative aspects of deoxyribose nucleic acid (DNA) metabolism in an amicronucleate strain of Tetrahymena . . . 71

MARUYAMA, K.
Contractile protein from crayfish tail muscle 95

HYMAN, LIBBIE H.
The occurrence of chitin in the lophophorate phyla 106

MBL/WHOI LIBRARY
WH 1AZJ S